Risk factors for treatment failure in isoniazid resistant tuberculosis

Thesis

RISK FACTORS FOR TREATMENT FAILURE IN ISONIAZID RESISTANT TUBERCULOSIS

by

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Photograph page 23

Image page 34

Photograph page 40

Photograph page 46

Photograph page 49

Table page 52

Photograph page 55

Photograph page 60

Image page 62

Photograph page 65

Table page 76

Image page 96

Table page 100

Image page 109

Image page 116

Image page 122

Table page 142

Image page 152

Image page 158

Abstract

There were 8.6 million cases and 1.3 million deaths from tuberculosis (TB) globally in 2012. Among the major challenges to global TB control and the ultimate goal of TB elimination is the increasing prevalence of drug resistant strains of *Mycobacterium tuberculosis* worldwide, coupled with an extremely limited pipeline of novel drug development. In 2012 there were an estimated 450,000 cases of muti-drug resistant TB (resistant to at least rifampicin and isoniazid) and 170,000 deaths due to multi-drug resistant (MDR) TB worldwide. The prevalence of resistance to isoniazid is extrememly high in some regions of the world, including Vietnam, where 25% of new smear positive patients and 54% of retreatment patients are infected with strains resistant to isoniazid. Treatment outcomes are known to be worse for patients with undiagnosed isoniazid resistant (INH^R) TB treated with standard regimens but the majority of patients have successful outcomes. This thesis investigated risk factors for treatment failure among patients with isoniazid resistant TB in Ho Chi Minh City, Vietnam.

Chapter one provides an introduction to tuberculosis and isoniazid resistant TB and chapter two describes the methodology of the studies decribed in the thesis. In chapter three, I investigate the treatment outcomes among a cohort of patients with isoniazid resistant tuberculosis treated according to National TB guidelines. The data show that unfavourable treatment outcomes are unacceptably high, at 19% among patients with INH^R TB.

In chapter four, I investigate three bacterial factors associated with unfavourable treatment outcomes among patients with isoniazid resistant TB, bacterial lineage, MIC to isoniazid and mutations responsible for isoniazid resistance. I show that the Beijing genotype is associated with young age, isoniazid resistance and multi-drug resistance, and unfavourable outcome.

In chapter five, I determine the prevalence of viral hepatitis co-infection among patients with INH^R TB. Infection with hepatitis B is 9.8% and hepatitis C 4.6% among patients with INH^R TB and is a risk factor for the development of anti TB drug-induced hepatitis (ATDIH) but not for unfavourable outcome.

In chapter six, I determine the distribution of acetylator types for isoniazid among Vietnamese TB patients and show that acetylator status is not a risk factor for unfavourable outcome in patients with INH^R TB.

Overall, these studies show that INH^R TB is a serious challenge to TB control efforts in Vietnam, with unacceptably high rates of treatment failure. In the final chapter, I discuss the implication of my findings and propose priorities for further research to address these issues.

Co-Authorship

The author of this work, Dr Phan Vuong Khac Thai (PVKT), was responsible for conduct, supervision, data analysis and writing of all studies described in this thesis.

Ms Nguyen Thi Hanh was responsible for administration of the study and liaison with the district TB units (DTUs). Dr Phung Khanh Lam provided assistance with data analysis.

Heads of 8 units including Dr Nguyen Thi Hong (out-patient department, PNT Hospital), Dr Nguyen Van Nghia (Phu Nhuan district), Dr Ngo Phuoc Duc (district 6), Dr Pham Thi Thuy Lieu (district 4), Dr Tran Ngoc Dai (Binh Thanh district), Dr Dao Cong Diep (district 1), Dr Nguyen Van Thom (district 8), Dr Hoang Van Thang (Tan Binh district) and staff were responsible for recruitment of patients, obtaining samples in accordance with the study protocol and follow-up and evaluation of the patient treatment outcomes.

Dr Dang Thi Minh Ha, Ms Nguyen Thi Bich Tuyen, Ms Vo Thi Ha and Mr Tran Van Quyet performed Microscopic Observation Drug Susceptibility (MODS) technique at PNT Hospital.

Dr Nguyen Ngoc Lan (Head of biochemistry laboratory), Ms Le Thi Ngoc Thanh (Head of nurse of biochemistry laboratory Pham Ngoc Thach Hospital) and staff conducted blood tests including liver function tests, viral serology for hepatitis B and C.

Dr Nguyen Thi Ngoc Lan (Head of microbiology laboratory), Ms Tran Thi Kim Quy (Head of nurse of microbiological laboratory Pham Ngoc Thach Hospital) and staff conducted homogenous sputum examination, sputum culture in the follow-up period and DNA extraction.

Ms Vo Sy Kiet conducted MAS PCR and spoligotyping and Ms Nguyen Thi Quynh Nhu conducted NAT2 testing and viral load at Oxford University Clinical Research Unit (OUCRU).

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Abbreviations

AD	Anno Domini
AFB	Acid fast bacilli
AIDS	Acquired Immune Deficiency Syndrome
ALP, AP	Alkaline Phosphatase
ALT	Alanine amino transaminase
AMK	Amikacin
Amx/Clv	Amoxycilline/Clavulanate
AntiHCV	Anti hepatitis C virus
ART	Anti-retroviral therapy
ARV	Anti-retroviral
AST	Aspartate transaminase
ATS	American Thoracic Society
ATDH, ATDIH	Anti-TB drug induced hepatotoxicity
AUC	Area under curve
A/G	Albumin/Globulin
BC	Before Christ
BCG	Bacillus Calmette Guerin
CDC	Centers for Disease Control and Prevention
Cfz	Clofazimine
Cfu	Colony forming units
Clr	Clarithromycin
CPM	Capreomycin
CPT	Cotrimoxazole Preventive Therapy
CRF	Case Record File
CRP	C-reactive protein
CR3	Complement Receptor 3
Cs	Cycloserine
CT	Computerized Tomography
CXR	Chest X-ray
DCs	Dendritic cells
DIH	Drug induced hepatitis
DILH	Drug-induced liver injury
DNA	Deoxyribonucleic acid
DOTS	Directly Observed Therapy Short course
DR	Direct Repeat
DR-TB	Drug-resistant tuberculosis
DST	Drug Susceptibility Testing
DTU	District Tuberculosis unit
DVR	Direct Variant Repeat
DVT	Deep Vein Thrombosis
EBA	Early bactericidal activity
ELISA	Enzyme-linked immunoassay
EMB	Ethambutol

ETH	Ethionamide
FIND	Foundation for Innovative New Diagnostics
FQs	Fluoroquinolones
GLC	Green Light Committee
GWAS	Genome Wide Association Studies
G+C	Guanine plus Cytosine
HAART	Highly Active Antiretroviral Therapy
HBeAg	Hepatitis B e antigen
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCMC	Ho Chi Minh City
HCV	Hepatitis C virus
HIV	Human Immunodeficency Virus
HLA	Human Leukocyte Antigen
IDR	Intradermal reaction
IDU	Intravenous Drug User
IFN-γ	Interferon-γ
IGRA	Interferon-γ Release Assay
IL	Interleukin
INH	Isoniazid
INH^R	Isoniazid resistant
INR	International Normalized Ratio
Ipm/Cln	Imipenem/cilastatin
KM	Kanamycin
LAB	Laboratory
LAM	Lipoarabinomanan
LJ	Lowenstein-Jensen
LM	Lipomannan
LPA	Line probe assay
LSP	Large Sequence Polymorphisms
LTBI	Latent tuberculosis infection
Lzd	Linezolid
ManLam	Mannose-capped Lipoarabinomannan
M.tb	Mycobacteria tuberculosis
MDGs	Millenium Development Goals
MDR	Multi-drug resistance
MGIT	Mycobacterial Growth Indicator Tube
MHC	Major Histocompatibility Complex
MIC	Minimal Inhibitory Concentration
MMP-1	Matrix metalloproteinase-1
MODS	Microscopic Observation Drug Susceptibility
MOTT	Mycobacterium other than tuberculosis
MTBC	Mycobacterium tuberculosis complex
NK cell	Natural killer cell
NAAT	Nucleic acid amplification test
NAD	Nicotinamide adenine dinucleotide

NAT-2	N-acetyltransferase-2
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NRAMP1	Natural resistance associated macrophage protein-1
NRTIs	Nucleoside reverse transcriptase inhibitors
NTM	Non-tuberculosis Mycobacteria
NTP	National Tuberculosis Control Programme
OUCRU	Oxford University Clinical Research Unit
PAS	P-aminosalicylic acid
PCR	Polymerase chain reaction
PCT	Procalcitonin
PDMI	Phthiocerol dimycocerosates
PGL	Phenolic glycolipids
PIs	Protease inhibitors
PMDRTB	Programmatic management of drug resistant tuberculosis
POA	Pyrazinoic acid
PPD	Purified protein derivative
PT	Prothrombin time
Pto	Prothionamide
PZA	Pyrazinamide
QFT	QuantiFERON
Rfb	Rifabutin
RFLP	Restriction Fragment Length Polymorphism
RiF	Rifampicin
SCC	Short-course chemotherapy
SD	Standard Deviation
SGOT	Serum glutamate oxaloacetic transaminase
SGPT	Serum glutamate pyruvate transaminase
SL	Sulfolipid
SM	Streptomycin
SNP	Single Nucleotide Polymorphism
TB	Tuberculosis
TBM	Tuberculosis meningitis
Thz	Thioacetazone
TDR	Totally drug –resistant TB
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Trd	Terizidone
TST	Tuberculin skin test
ULN	Upper limit of normal
USA	United States of America
UV	Ultra violet
VM	Viomycin
VNTR	Variable Number Tandem Repeat
V/Q scan	Ventilation/Perfusion scan
WHO	World Health Organization
XDR	Extensively-drug resistant
ZN	Ziehl-Neelsen

Table of Contents

List of Figures

List of Tables

Chapter 1

Introduction

1.1 Tuberculosis:

1.1.1 History:

Tuberculosis (TB) has been with us for thousands of years. TB is an infectious respiratory disease and a classic disease of poverty and as such is more common in underdeveloped regions of the world because of differences in culture, politics and socioeconomic development [1]. Historical evidence of TB has been identified via pathology in ancient mummified human remains and molecular techniques have been used to confirm the presence of *Mycobacterium tuberculosis (M. tb)*, the bacterium which causes TB disease [2]. Historical evidence of TB also comes from both primary and secondary accounts.

It was long thought that humans contracted TB from infected animals, probably cattle, when they were domesticated about 10,000 years ago. However, this theory has now been disproven through phylogenetic analysis of mycobacterial species, which suggests bovine TB caused by *Mycobacterium bovis* (*M.bovis*) evolved from *Mycobacterium tuberculosis* and hence man probably gave TB to cattle following domestication [3, 4].

Evidence of TB can be found in the Old World dating back thousands of years such as in Italy (5800 BC). In 400 BC, The Greek physician Hippocrates first described the clinical symptoms of TB using the term 'phthisis'. In Asia, China has evidence of TB

in a mummy dated to between 206 BC and the seventh century AD, while Japan and Thailand have possible evidence dated to the seventh century AD and 300 BC-300 AD, respectively. The earliest evidence of skeletal TB in the New World may be in South America [5]. Pictorial evidence comes in a variety of forms including painting, drawings, reliefs and sculptures. Figure 1.1 shows an example of a two thousand year old sculptural representation of a woman with TB from Colombia.

Figure 1-1 : Representation of a woman with pronounced gibbus (Pott's disease?). Momil culture, 200 BC to 100 AD, Sinu River, Colombia (reproduced from Sotomayor 1992) [6]

There are large bodies of written and illustrative evidence that have contributed to tracing the evolution and history of this infectious disease. A Chinese text (2700 BC) provides a description of hemoptysis and TB in the cervical lymph glands [7]. Ebers Papyrus (1500 BC) also describes TB of the lymph glands[8].

In the sixteenth century, Fracastorius (1483-1553) wrote "De Contagione" and suggested that TB was due to invisible "germs". By the mid-1600s, 20% of deaths in England were due to TB.

In the eighteenth century, John Bunyan referred to TB as the " Captain of all these men of death" [9]. By the beginning of the nineteenth century, TB was the leading cause of death in most European countries.

From the beginning of the nineteenth century, many advances in diagnostics, treatment and bacteriology of TB were made. In 1816, Rene Laennec invented the stethoscope and three years later he published his book named " De l' Auscultation Mediate". In this book, he described his new method of diagnosis using the stethoscope. Sadly, he himself died of pulmonary tuberculosis in August 1826. Carlo Forlanini, born in 1847, developed the first specific treatment for pulmonary tuberculosis: artificial pneumothorax in 1906. Jean Antoine Villemin proved in 1867 that tuberculosis was an infectious disease transmitted by contact from humans to animals and from one animal to another [10]. On March 24, 1882 a major breakthrough was achieved when Robert Koch made his famous presentation, 'Die Aetiologie der Tuberculosis' in which he presented demonstrations of the tubercle bacillus. He was awarded the Nobel Prize in Medicine or Physiology in 1905 for this work and World TB day is now marked on 24th March each year in honour of the event. In 1890 Koch isolated a substance from tubercle bacilli which he thought could stimulate immunity to the pathogenic bacteria and cure TB patients. He called this substance tuberculin. Later studies proved that Koch was unfortunately mistaken in

his claims for tuberculin, but it continues to be used in the tuberculin skin test for TB diagnosis.

In 1895, Conrad Roentgen discovered X- rays which provided a new method of diagnosing TB. The theory of transmission of TB via droplet infection was established in 1897 [10]. By the mid -nineteenth century, the concept of the sanatorium with fresh air, a good healthy diet, rest and graded exercise for TB treatment had been established.

Also from the late nineteenth century, many organizations were established to combat TB. In 1889 the Tuberculosis Association was established in the USA and in 1890s the League Against Tuberculosis was founded in France. One year later, the National Association for the Prevention of Tuberculosis and other forms of consumption was established in Britain[8]. In 1902, The International Union against TB was founded. It was set up to encourage a system of control including the notification of all cases, contact tracing and the provision of dispensaries and sanatoria. As the result of the work of these organizations and advances in understanding of the disease, coupled with improvements in sanitation and living standards across Europe, by the second half of the nineteenth century and into the twentieth, there was a marked decline in TB in Europe and the United States.

At the begining of the twentieth century, the field of immunology began to develop. The Viennese peadiatrician, Clemens von Pirquet, had described 'serum sickness' using the terms allergy and allergen in 1903. Two years later he recognized that positive tuberculin reactions reflected latent infection with *M.tb* and proposed a cut-off point of 5 mm for a positive skin test. Charles Mantoux introduced the use of a

cannulated needle and syringe to inject tuberculin intracutaneously in 1908. Also in this year Calmette and Guerin began developing their TB vaccine. Florence Seibert developed standardized purified protein derivatives (PPD) which allowed more studies of tuberculin reaction sizes. In 1921, after years of attenuation by *in vitro* passage, the bacillus Calmette Guerin (BCG) BCG vaccine was introduced and distributed generally by 1924. From 1860 to 1945, Vietnam was under French colonial rule and so BCG was used extensively in Vietnam as early as the 1920s as part of the French health policy to fight TB. Although BCG vaccine has been used for a long time as a preventive measure, its protective effects have widely varied in evaluation, ranging from 80% to a negative effect. The reasons for this observed variation in protective efficiency are unknown, but several hypotheses have been proposed, including variations in the vaccine strains used in different regions of the world, variation in prevalence and exposure to environmental mycobacterial species and differences in the prevailing strains of *M.tb* [11]. However, the protective effect of BCG vaccine against disseminated TB in children has been consistently evident [12].

The incidence of TB death has declined with improvements in living standards, control strategies and the development of antituberculous drug therapy. In 1944, Waksman introduced the antibiotic streptomycin, which was to be the first effective drug treatment for TB. After that, in 1945 Trudeau established the first truly successful sanatorium in the US for the open-air treatment of tuberculosis [10]. In the 1950s BCG vaccination, health education and pasteurization of milk all contributed substantially to TB control.The introduction of streptomycin was quickly followed by

para-amino salicylic acid (PAS) in 1946 by Lehmann, isoniazid in 1952 and rifampicin in 1963. The modern era of tuberculosis treatment and control was established. Treatment to cure became a realistic goal for every patient in the world [13].

As the modern era of TB treatment began, drug resistance emerged. Monotherapy for TB results in the rapid selection of spontaneously occurring mutant bacilli. Resistance to streptomycin was shown shortly after its first use in patients [14-16]. Multi-drug therapy with at least three effective agents is required to prevent the selection of resistant strains. Inadequate therapy due to prescription or adherence, co-morbidities leading to poor absorption and other factors can lead to the amplification of resistance to more than one drug. For *M.tb* resistant to at least the two most potent drugs, isoniazid and rifampicin, treatment is more difficult and mortality rates are higher, therefore these strains are termed multi-drug resistant (MDR) TB [17]. In the late 1980s TB was thought to have been controlled and moving towards eradication in the developed countries but TB has since resurged with the rise of drug-resistant strains and the emergence of the HIV pandemic [18, 19].

In 1990, the Directly Observed Therapy Short-course (DOTS) strategy was developed by WHO and introduced to endemic countries with five essential components: (1) microscopic examination of sputum for acid-fast bacilli (AFB), (2) use of rifampin-based regimens, (3) reliable supply of high-quality drugs and diagnostics, (4) reporting of treatment outcome and monitoring of programmes, and (5) political commitment from governments.

Initially implemented in just ten countries, by 1998, 119 countries had implemented the DOTS strategy [20]. In 1993, The World Health Organization declared TB a global emergency, estimating that one third of the world population are infected with *M.tb.* As TB case rates decreased in developed countries, funding for research and implementation also declined although TB remained the leading infectious disease in the world, mainly in underdeveloped countries.

Together with HIV infection fanning tuberculosis, MDR TB emerged. An outbreak in the United States in the late 1980s and early 1990s drew attention to the problem of emerging drug resistance which was going undetected, untreated and transmitting within communities. In the USA and in the developed countries, diagnosis with culture and drug-susceptibility testing was available to enable treatment with appropriate second-line anti TB drugs but in resource-limited settings, MDR TB treatment was prohibitively expensive. However, in Peru, a high-burden developing country, the strategy of MDR TB treatment based in the community was initiated with good results. By the end of the 1990s, the Green Light Committee, together with the help of the Bill and Melinda Gates Foundation began to encourage and help Peru and other countries in the programmatic management of MDR TB treatment (initially called DOTS-Plus).

At the millennium a set of targets for international development were agreed by international agencies and Non-Govermental Organizations (NGOs) known as the Millenium Development Goals (MDGs) such as reducing extreme poverty, improving sources of drinking water and maternal/child health [21]. For TB, The Stop TB Partnership has presented two specific goals: to halve TB prevalence and to halve TB

mortality by 2015 compared to these two levels in 1990. Recently the additional goal of TB eradication by 2050 was also added (defined as a global incidence of less than 1 per million population). Although achieving the MDGs is challenging, especially in some regions like Africa and Eastern Europe, significant progress has been made. In 2013, WHO's Stop TB Department set targets for the post-MDG era- annual deaths would be halved by 2025 and TB incidence would drop from 16 to 10% in the next ten years. TB eradication will require the development of significantly improved new effective drugs, diagnostics and vaccines. Today, rapid nucleic acid-based tests for drug-resistant TB are available and several new drugs are in the development pipeline, with the first new drug class being approved by the FDA and WHO for MDR TB in 2013 [22]. Funding and political will need to be sustained to maintain momentum in the drive towards TB eradication, now and in the future [23].

In the past, TB has increased with population size, bad sanitation, poverty, trade and emigration. On the other hand, TB has also declined with improvements in living conditions, improved nutrition, better diagnosis, health education, vaccination, isolation of people with TB, and curative treatment. The major challenges to TB control today are the HIV/AIDS pandemic, antibiotic resistance, poverty and political instability.

1.1.2 Global epidemiology of tuberculosis:

Tuberculosis is an infectious disease caused by *M.tb*. It is principally transmitted from one person to another via droplets in sputum expectorated from the respiratory tract of individuals with active tuberculosis disease.

An untreated sputum smear-positive case infects about 10 other individuals each year [24]. Around 5% of those infected with *M.tb* develop progressive primary disease in the 1-2 years following infection. The proportion is higher in vulnerable groups such as HIV infected and immunocompromised individuals, children and the elderly [25]. Mortality from TB is high in the absence of effective treatment. Evidence from the pre-chemotherapy era suggests around 60% of cases will be fatal without treatment. The remaining third will either remain chronically ill or recover from the disease but in most cases continue to harbor a latent infection. Among smear-positive patients receiving antituberculosis drugs, the case-fatality rate can exceed 10% within 1 year, due to factors such as late diagnosis, severe complications, drug resistance or drug reactions [5].

Tuberculosis is predominantly a disease of adults. Although children 0-14 years make up 30% of the world population, they account for only 10% (9.6%-11%) of incident cases with the majority of cases occurring in high TB burden countries. This data may be an underestimate because of many challenges and difficulties in estimation of TB burden in children like the lack of definite diagnosis, the presence of extra-pulmonary TB which requires specialist to detect, and the lower public health priority [26-28]. Where transmission rates are high, TB incidence peaks in young adults [29]. In industrialized countries, as transmission falls, the average age of TB cases increases [29]. The majority of indigenous TB cases in developed low-incidence countries are found among the elderly due to reactivation of latent infection and subsequent transmission within care settings. Globally, TB is more common in men than women in the ratio of 2:1 and it remains a major cause of death among men in endemic

countries along with alcoholism and cardiovascular disease. Around two-thirds of cases are estimated to occur among people aged 15-59 years, the economically productive age-group [30].

The HIV pandemic has had a dramatic impact on the TB pandemic [31]. HIV infection, results in a gradual decline in T-cell immunity which is the crucial immune component in control of *M.tb* infection. Therefore HIV infection dramatically increases the risk of TB disease following primary infection and of reactivation of latent TB infection [32]. Other factors known to increase the risk of TB include diabetes, silicosis and malnutrition and exposure to smoke from smoking or biomass fuel [33]. An association between smoking and tuberculosis has been shown in numerous studies, with a strong dose-response relationship. People smoking more than 15 pack-years had the highest risk in one study (adjusted OR 1.90, 95% CI 1.28 to 2.81) [34]. Overcrowded living conditions, particularly in urban areas increase the risk of TB transmission. The chronobiological aspects related to TB such as seasonality, latitude, photoperiod, radiation, associated infections and circannual oscillations of lymphocytes activity, may be relevant in some settings but the relationships are not well characterized. More research is required to elucidate these relationships [35].

It has long been well-known that "TB runs in families". After infection with TB, individuals have differences in outcome due to variations in innate host defense mechanisms like immune recognition, phagocytosis, cytokine production and effector mechanism in addition to environmental influences such as malnutrition. Several gene polymorphisms have been found to be associated with increased susceptibility and

severity of TB disease [36]. Further details are discussed in the TB pathogenesis section 1.1.4.

As the second leading cause of death from infectious diseases worldwide (after HIV/AIDS), TB remains a major global health problem (figure 1.2). In 2010, there were an estimated 8.8 million incident cases of TB [range: 8.5-9.2 million]. The absolute number of TB cases as well as TB incidence rates have been falling gradually since 2002. There were 5.7 million notifications of new and recurrent cases of TB (65% of the estimated number of incident cases in 2010). Most of these cases were in Asia (59%) and Africa (26%). The proportion in other regions were: Eastern Mediterranean Region (7%), Europe (5%) and America (3%). The four countries with the largest incident cases were India, China, Indonesia and Pakistan. TB prevalence in 2010 was estimated at 119 (113-135) per 100 000 population [30].

The majority of TB patients worldwide are now treated in programmes with DOTS/ Stop TB Strategy with successful outcomes. At a global aggregate level, the treatment success rate, under WHO definitions for patients diagnosed and treated under DOTS was 87% among new cases of smear-positive pulmonary TB in 2009. In 2010 there were 1.1 million [range, 0.9-1.2 million] deaths among HIV-uninfected individuals with TB and approximately an additional 0.35 million [range: 0.32-0.39] deaths among HIV-coinfected TB patients. The Stop TB Partnership (www.stoptb.org) of the WHO has set targets for 2015 aiming to reduce prevalence and death rates by 50% compared with 1990. For 2050 the goal is to reduce the global incidence of active TB cases to under 1 case per 1 million population per year, the recognized definition of TB 'elimination' [30].

WHO has also addressed the issue of co-epidemics of TB and HIV. In 2010, globally 34% of TB patients were tested for HIV, and about 13% of TB cases were found to occur among HIV– infected individuals. 80% of people with HIV were started on cotrimoxazole preventive therapy (CPT) and 46% on antiretroviral therapy (ART). WHO-initiated Global Plan targets aim that all TB patients will be tested for HIV and all TB patients with HIV will be treated with CPT and ART [30]. Significant progress has been made in integrating TB-HIV control activities in many regions but funding, politicial commitment and human resource training are all essential to maintain momentum in progress.

Figure 1-2 : Estimated TB incidence rate 2010 from Global Tuberculosis Control 2011, WHO[30]

1.1.2.1 Epidemiology of Tuberculosis in Vietnam:

Tuberculosis control activities in Vietnam started in 1957, with separate systems in the North and the South. After the reunification of Vietnam in 1975, the National Institute for tuberculosis in Hanoi established national guidelines for tuberculosis control as well as for specialized clinical care for the whole country. A new national tuberculosis control programme (NTP) was introduced in 1986 organized according to principles laid down by International TB Control Association [37] .

Tuberculosis control activities in Vietnam expanded rapidly following establishment of the programme. The proportion of districts covered by the NTP reached 100% by the year 2000 [37]. Within districts, training of communal health workers and provision of supplies expanded so that the proportion of communes applying NTP guidelines increased from 18% in 1986 to 99% in 2000. Short-course chemotherapy (SCC) using two months treatment with streptomycin, rifampicin, isoniazid and pyrazinamide, followed by six months with isoniazid and ethambutol (2SRHZ/6HE) was first introduced in 1989 in 12 districts of 4 provinces and was subsequently made available in all districts in 1999. The national notification rate for new smear-positive patients has fluctuated. Notification increased from 11 per 100 000 population in 1986 to a peak of 73 in 1998 following expansion of case detection and then began to decrease slightly to 69 in 2000 and 59 in 2010. Notification rates are higher in the South than in the North and remain low in the mountainous provinces. The proportion of new tuberculosis cases detected by the programme was stable, at around 60% in the years 1991-1995, increased to 80% in 1996, reached 86% in 1998 and declined again

slightly to 83% in 1999 and 81% in 2000. Vietnam was thought to be the first high-burden countries to reach the WHO targets of 70% case detection rate and 85% cure rate but despite this TB rates have not fallen significantly and are only now beginning to decline at approximately 1-2% per year as Vietnam develops into a middle income country [38].

Recently the case detection rate has been revised downwards to just 54%. This re-estimation was following a prevalence survey in 2006-2007 which showed that previous estimates of the case burden in Vietnam were too low, resulting in an over-estimation of the case-detection rate. The first national survey of tuberculosis prevalence in Vietnam was conducted in 2006-2007 and this study was published in the Bulletin of the World Health Organization in 2010 [39]. The survey showed a weighted prevalence rate of smear-positive tuberculosis among the population aged > 15 years of 196.8 per 100 000 population, which was 1.6 times as high as previously estimated by WHO. The NTP authorities suggested some additional approaches to TB control such as 1) introduction of active case-finding based on chest X-ray screening in high-risk groups, and 2) widening of the eligibility criteria for smear examination to other symptoms in addition to cough [39]. SCC cure rates have been stable at 80%-90% from 1994 to 2000 and 92% in 2009 [37].

Many factors have contributed to the successful implementation of tuberculosis control in Vietnam. Strong political commitment is the first factor and probably, the most important one. Tuberculosis control is a part of the Comprehensive Poverty Reduction and Growth strategy and contributes to the attainment of the Millennium Development Goals for poverty reduction in Vietnam. Tuberculosis control activities

at district and commune levels are fully integrated into the general health care system. In addition to government support, the NTP has received major sustained technical and financial assistance from international organizations.

Vietnam remains among the 22 high burden countries for TB, ranking twelfth in the list. In 2010, TB prevalence was 334 (147-576) per 100 000 population (including HIV infection), and the percentage of new TB cases with MDR was 2.7 (2.0-3.7) [30]. The NTP is facing several challenges such as a rapid increase in the proportion of TB cases with HIV co-infection, MDR and XDR TB, TB in prisons, in elderly and in psychiatric patients as well as combating TB in the general population. A sustained decline above the current rate of 1% per year will be required to have a real impact on the TB epidemic in Vietnam.

1.1.3 *Mycobacterium tuberculosis*:

M.tb complex belongs to the genus Mycobacterium. *Mycobacterium leprae* which causes leprosy is the only other major pathogen in this group. Other mycobacteria are variously termed the 'atypical mycobacteria', 'non-tuberculous mycobacteria' (NTM) or 'mycobacteria other than tuberculosis' (MOTT). NTM will be used in this thesis. The *M.tb* complex includes *M.tuberculosis, M.bovis, M. africanum, M. canetti, M.microti, M.caprae* and *M.pinnipedi* [40] (table 1.1). The cell wall of *M.tb* lacks a phospholipid outer membrane, therefore *M.tb* is sometimes described as a Gram-positive bacterium. However, *M.tb* stains only weakly Gram-positive because of the high lipid and mycolic acid components in the cell wall [41] . In smears stained with the acid-fast stain technique, the tubercle bacilli typically appear as straight or slightly

curved rods. *M tb* is a small, aerobic, non-motile and non-sporulated bacillium. The dimensions of the bacilli are 1-10μm in length (usually 3-5μm) and 0.2-0.6 μm in width (figure 1.3).

Category	**subgroup**
Kingdom	Bacteria
Phylum	Actinobacteria
Class	Actinobacteria
Subclass	Actinobacteridae
Order	Actinomycetales
Suborder	Corynebacterineae
Family	Mycobacteriaceae
Genus	Mycobacterium
Species	*M. tuberculosis* *M. bovis* *M. africanum* *"M. canettii"* *M. pinnipedii* *M. microti* *M. caprae*

Table 1-1: Taxonomic classification of the agents of TB

Under favourable laboratory conditions, *M.tb* divides every 16 to 20 hours [42]. The cell wall characteristic of impermeability limits nutrient uptake and so slows the growth rate. The long generation time results in slow *in vitro* culture and explains the sub-acute or chronic characteristic of disease. *M.tb* is also able to survive very low temperatures. The bacilli are sensitive to heat, sunlight and ultraviolet (UV) irradiation. *M.tb* tolerates low oxygen tension and may survive for many years in this condition but in a dormant state under oxygen stress[43] .

M.tb has a plasma membrane, a cell-wall and an outer capsule-like layer. The cytoplasmic membrane of mycobacteria contains lipopolysaccharides with functions including osmotic protection, traffic regulation of specific solutes and the cell house-keeping tasks. The membrane also contains proteins with different functions such as sensing of the concentration of molecules in the environment, translocation of signals to genetic and metabolic machinery in the cytoplasm. The membrane also contains enzymes which are involved in metabolic processes and mediate selective passage of nutrients and ions. These proteins in the plasma membrane of *M.tb* are potential novel drug targets, diagnostic probes or components of a vaccine against tuberculosis [44].

The cell wall protects the cell contents and forms the structural shape of the bacterium. Proteins in the cell wall synthesize cell wall components and porins allow diffusion of aqueous solutes. Peptidoglycan forms the structure shape of the cell wall with covalently bound arabinogalactan, a brached polysaccharide, whose outer ends are esterified with mycolic acid, which is a unique high molecular weight fatty acid. The mycolic acids are responsible for several of the physiological aspects and mechanisms of TB disease [45] .

The outer layer of the cell wall presents an array of free lipids, such as phthiocerol dimycoserosates (PDMI), phenolic glycolipids (PGL), trehalose-containing glycolipids and sulfolipid (SL). Traversing the whole envelope, some glycolipids such as phosphatidyl-myo-inositol mannosides, lipomannan (LM) and lipoarabinomanan (LAM), are connected to the plasma membrane and extend to the exterior of the cell wall. LAMs are species-specific and function to modulate the host immune response and anti-inflammatory effects. ManLam (mannose-capped LAM) has been demonstrated as powerful anti-inflammatory molecules and key virulence factors [46].

The structure of *M.tb* gives it some characteristics such as acid fastness, extreme hydrophobicity, impermeability, resistance to injury and distinctive immunological properties. The tubercle bacillus is prototrophic and heterotrophic.

Figure 1-3: Electron microscopy of *Mycobacterium tuberculosis* growing in culture from www.wadsworth.org

1.1.4 Pathogenesis of Tuberculosis:

The clinical phenotype of tuberculosis disease results from the exposure dose and route and the subsequent interaction between the bacteria (*M.tb*) and the human host immune response.

Mycobacteria are inhaled in droplet nuclei which are small enough to bypass the mucociliary defences of the bronchi and can pass into the terminal alveoli of the lungs where they encounter cells of the innate immune system, triggering a complex series of events with three possible outcomes. The first one is the elimination of the bacteria completely, the second is the containment of the infection, usually in a granuloma, for a prolonged period (latent TB), and the last one is the immediate progression to active disease with clinical manifestations. In the case of latent TB it is still unclear whether the bacilli are in a state of reduced metabolic activity or completely dormant in a spore-like form that is metabolically inactive awaiting a signal to resume division. However, some studies show that latent bacilli may be metabolically active because they remain sensitive to chemoprophylaxis [47] but the population may not be homogenous and may represent a spectrum of metabolic states.

Once a person is infected with *M.tb*, the chance of developing active disease is greatest in the first year following infection, declining exponentially thereafter [5]. The risk is greatest in the immunocompromised including young children, the elderly and advanced HIV. Children under 5 years of age represent around 50% of all pediatric cases. Within the first year of infection, the incidence of clinical disease is approximately 1.5%, and the cumulative risk during the first 5 years is estimated to be from 5 to 10% [5]. Post-primary TB may occur after months or years without clinical

signs following primary infection. The emergence of the disease is due to the reactivation of dormant bacilli which may be in response to a weakening of the immune system, but the factors leading to disease reactivation in the absence of overt immunosuppression are not well understood. Post-primary TB generally occurs in adults. Reinfection of a person who has had a previous primary infection may also lead to active TB, particularly in high-risk congregate settings, such as prisons in high-burden countries [48-50].

In the majority of individuals infected with *M.tb,* the immune system is able to contain the infection with pulmonary lesions gradually healing. For the remaining 10% of individuals the environment is favourable for the bacilli and their multiplication continues resulting in a primary lesion called the Ghon focus.

After *M.tb* bacilli enter the lung, they interact with neutrophils which, however, are unable to eradicate the infection and a signaling cascade is triggered which attracts macrophages to the site of infection.

Various receptors on the surface of macrophages and dendritic cells (DCs) are able to recognize *M.tb,* including toll-like receptors (TLR), complement receptors, mannose receptors, and scanvenger receptors. These receptors recognize various components of *M.tb* [51] and trigger pro-inflamatory signalling which activates other macrophages to ingest and digest the invading *M.tb* in addition to recruiting T-lymphocytes to the infected area. The macrophages present the *M.tb* antigens on their surface in association with HLA Class II antigens. If the bacilli successfully evade the initial intracellular destruction via phagosome-lysosome fusion and production of reactive oxygen and nitrogen species, the bacteria will be able to multiply and the

macrophages will be disrupted [52]. Elimination of *M.tb* infection is thought to be highly dependent on the interaction between macrophages and lymphocytes. Dendritic cells transport antigens from phagocytosed bacterial cells at the primary Ghon complex to the proximal draining lynph nodes, where they express presentation molecules in high density to activate naïve T-lymphocytes, such as major histocompatibility complex (MHC-I or II) , as well as co-stimulatory molecules, such as IL-12, IL-18, or IL-23 [53]. At this stage, the bacilli may disseminate from the primary complex via a transient bacteraemia leading to the development of disease at sites outside the lungs, most commonly in the cervical lymph nodes but any organ of the body may be affected. CD4+ T-lymphocytes are known to be crucial in the control of *M.tb* infection, as evidenced by the dramatic increase in susceptibility to TB among people with advanced HIV infection. Activated T lymphocytes appear rapidly after infection and recognize *M.tb* antigens.

CD4+ lymphocytes may develop into either Th1 or Th2 cells with different patterns of cytokine secretion and the Th1/Th2 balance is important in determining the outcome of infection. CD4 T cells provide the crucial protective effect by the production of gamma interferon (INF- γ). The role of other T-cell subtypes like CD8 T cells is incompletely understood, but the Th1/Th2 balance must favour TH1 for protective efficacy at this stage [54, 55] .

Two or three weeks after infection, T-cell immunity develops with the presence of antigen-specific T lymphocytes. These T lymphocytes will proliferate and activate macrophages to kill mycobacteria [53]. Central solid necrosis may inhibit the bacilli at this stage resulting in a dormant infection. Disease may progress directly after

primary infection or, within 2 - 3 years, disease (post primary TB) develops due to immune failure. The final outcome of TB infection depends on the balance between growth and killing of *M.tb*, on tissue necrosis, and fibrosis or regeneration.

1.1.5 Pathology of Tuberculosis

TB is one of the granulomatous inflammatory conditions (figure 1.4). The basic inflammatory response to infection by *M.tb* is the formation of a granuloma that is the pathologic hallmark of TB. A granuloma comprises a microscopic aggregation of activated macrophages (epithelioid) surrounded by lymphocytes. There are many types of cells which participate in the formation of a granuloma such as epithelioid cells, Langhans giant cell or foreign body giant cells, lymphocytes, polymorphs, plasma cells, eosinophils and fibroblasts. Granuloma formation is a complex procedure involving many various cells and immune effectors like chemokines and cytokines [56]. Granuloma formation is a part of adaptive immunity, however, innate immune cells are significantly involved.

The macrophages activate and develop into epithelioid cells with abundant pale-pink cytoplasms, indistinct borders, and vesicular nucleoli [57]. This type of cell has a secretory function to enhance the microbiocidal properties and induce necrosis of lung tissue. Epithelioid cells may fuse to form Langhans giant cells which have various configurations and can be at the periphery or anywhere in a granuloma [57].

The granuloma provides the environment in which the activated macrophages can inhibit the growth of *M.tb*. Bacteria can become dormant when they are in a granuloma and this situation is called latent TB. However, when immunity fails or *M.tb* replicates, reactivation of TB occurs. If the macrophages undergo apoptosis, this

leads to caseous necrosis which is described as firm, pale, crumbly and yellowish tissue like soft white cheese and leads to the spread of TB infection. The development of caseous necrosis in a TB granuloma correlates with pathogenic dysregulation of lipid metabolism [57]. According to Elkington and more recent studies, *M.tb* drives the expression of matrix metalloproteinease-1 (MMP-1) which can degrade type I collagen and drives the destruction of pulmonary tissue in TB [58].

Figure 1-4 : Definition of the stages of human TB lung granuloma

Reproduced from Kim et al. EMBO Mol Med. 2010 [57]

A,B. nascent granuloma (A) (x40) and nascent granuloma (B) (x100)

C,D. caseous granuloma (C) (x100) and caseous granuloma (D) (x40)

E,F. fibrocaseous granulomas (x40), and

G,H. resolved granulomas (x40)

1.1.6 Host genetic susceptibility to TB

It has long been recognized that certain ethnicities or families appear to have a high level of susceptibility to TB and it was at one time widely thought to be a heritable condition. The genetic basis of this susceptibility has been traced to polymorphism in many genes and TB susceptibility is now known to be polygenic. One of the first host susceptibility genes for TB was first identified in mice. This gene was initially called *Bcg* then renamed Natural Resistance-Associated Macrophage protein-1 (NRAMP1) and finally termed SLC11A1, the gene which encodes the protein, although it is still often referred to as NRAMP [59, 60]. Many other genes, including those for vitamin D receptors and the components of IFN-γ signaling pathways have also been identified as having a role in susceptibility to tuberculosis, although the complexities of the polygenic interactions resulting in susceptibility to active disease have yet to be unravelled [61]. Genome Wide Association Studies (GWAS) has been applied to many infectious diseases including TB but the complexities of polygenic susceptibility have yet to be fully elucidated [62, 63]. GWAS have been conducted on the Vietnamese population, and the results may suggest pathways and genetic factors leading to increased risks of TB in this population. Vietnam has participated in the 1,000 genomes project (www.1000genomes.org) which aims to map human genetic polymorphism across ethnicities and strengthen host genetic disease susceptibility research.

1.2 Pulmonary Tuberculosis :

1.2.1 Symptoms and Signs:

Primary Tuberculosis (TB) can be detected at the asymptomatic stage but in high-burden settings this rarely occurs, while in developed countries diagnosis is often made by Chest X-ray (CXR) during health-screening or contact tracing. A history of TB contact is the single most important factor in non-endemic settings and the conversion of the tuberculin test from negative to positive is an evidence for primary TB, although this is rarely documented outside developed countries. The patient may present with symptoms of upper respiratory tract infection such as cough, malaise but weight loss is uncommon. Enlargement of the lymph node may cause complex obstruction resulting in collapse of the peripheral lung leading to pneumonia with symptoms of cough, fever and shortness of breath. Permanent damage to the lung may occur resulting in bronchiectasis. From primary infection, a number of manifestations of extrapulmonary TB may occur such as TB pleural effusion (after 6-12 months), miliary or meningeal TB disease (within 6-12 months). Dissemination to other distant sites like bone or genitourinary tract may also occur during bacteraemic 'seeding'.

Two common manisfestations of primary TB are erythema nodosum and phlyctenular conjunctivitis. Erythema nodosum appears as reddened or brown swellings that usually have a dimension of 3-18mm. They are on the extensor surfaces of the limb, most commonly on the shins and sometimes on the upper limbs (figure 1.5). Arthralgia occurs in more than 50% of patients. It is caused by perivascular inflammation of the arteries and veins within the dermis. High circulating levels of immune complexes are probably responsible for this manifestation. Differential

diagnoses are sarcoidosis, streptococcal throat infection or inflammatory bowel disease. It may be idiopathic in 70% of cases. Erythema nodosum caused by TB usually occurs 3-8 weeks after TB infection. Lesions usually resolve with TB treatment although some discoloration of the skin may remain permanently [64]. Phlyctenular conjunctivitis is uncommon and it may occur in children usually within 12 months of primary infection. Lesions appear as small grey or yellow nodules on the conjunctiva near the limbus with dilated vessels radiating outwards. It may have symptoms like irritation, pain, lacrimation and photophobia. Lesions can appear and disappear spontaneously. Treatment of TB and local therapy such as atropine and hydrocortisone drops for relieving inflammation are the rule for resolution.

Figure 1-5 : Erythema nodosum.

Reproduced from http://www.patient.co.uk/doctor/erythema-nodosum.

Individuals with post-primary TB may be asymptomatic and be detected only by screening or on routine chest radiography. The commonest symptoms of TB are cough and prolonged sputum production (> 2 weeks). Other lung symptoms which may occur are haemoptysis, breathlessness, chest pain and constitutional symptoms such as malaise, tiredness, fever, weight loss, night sweats, pallor and anorexia. Clubbing may be present in extensive or long-standing disease. Respiratory signs may be absent, especially in the early stages. There may be evidence of consolidation, reduced breath sounds and crackles. Wheezes may be present if obstruction is present. Amphoric breathing may be heard. In chronic disease, the trachea and mediastinum may be deviated toward the side of disease.

1.2.2 Diagnosis of tuberculosis

The diagnostic approach for possible tuberculosis includes a detailed medical history, clinical examination, and if available, radiological, microbiological, immunological, molecular and histological examination. Definitive diagnosis of pulmonary TB requires the identification of *M.tb* in culture of sputum samples. According to WHO guidelines, if suspicion of pulmonary TB is high despite culture and smear for *M.tb* being negative, six sputum samples and two different chest radiographs over a period of 2 weeks should be performed to confirm the diagnosis of sputum-negative pulmonary TB [65]. In practice, ruling out TB is difficult and resource intensive in these circumstances, mycobacterial culture may not be available and many clinicians will initiate treatment rather than leave a potential TB case untreated.

American Thoracic Society (ATS) definitions for classification of suspected TB cases are as follows:

No TB exposure and no TB infection : patient has no history of exposure to TB subject and has a negative reaction to the tuberculin test.

TB exposure and no evidence of TB infection : patient has a history of TB exposure but has a negative reaction to the tuberculin skin test.

Latent TB, no TB disease : patient has a positive reaction to the tuberculin skin test but has no clinical, bacteriological or radiographic evidence of active TB.

Clinically active TB: patient must have clinical, bacteriological and/or radiographic evidence of current tuberculosis [66].

1.2.3 Differential diagnosis

There are many diseases that need to be considered in the differential diagnosis of pulmonary TB. A list of potential diseases for differential diagnosis is summarized in table 1.2:

Table 1-2 : Differential diagnosis (from Clinical Tuberculosis) [5]

*** Usually unilateral cavitation. Often occurs in the presence of other pulmonary diseases such as bronchiectasis or chronic obstructive pulmonary disease**

Routine blood tests including total blood cell count, liver function tests (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and total bilirubin), kidney function tests (urea and creatinine), blood glucose, electrolytes and uric acid, should be obtained before TB treatment. It is recommended all patients with TB are tested for HIV infection and HIV/TB service integration has been scaled up in recent years in many countries but the goal of universal coverage is yet to be achieved [67].

1.2.4 Smear microscopy

Sputum samples should be examined for acid fast bacilli using smear microscopy[68]. The high lipid content of the cell wall of mycobacteria renders them resistant to decolorisation by acid-alcohol after primary staining with basic fuchsin and hence acid-alcohol fast bacilli can be visualized with high specificity. Ziehl-Neelsen staining is the most widely used technique [69]. The sputum specimen should be first concentrated by centrifugation or filtration to improve the sensitivity of the test, but the majority of smears performed worldwide are direct smears (unconcentrated sputum) due to resource limitations. Alternative, simplified concentration methods, such as by magnetic beads, have been developed but none is yet in wide-spread use [70-73]. The specimen is spread onto a microscope slide then heat-fixed, stained with the primary stain (usually carbol fuschin), heated to enhance penetration of the dye into the bacilli, decolorized with acid-alcohol solution and finally counterstained with a dye such as methylene blue in order to obtain better differentiation between the acid fast bacilli and the background. A modification of Ziehl-Neelsen stain known as the Kinyoun stain, allows cold acid-fast staining. Fluorescent staining, usually with

auramine O as the primary stain, allows more rapid reading of slides but can result in reduced specificity due to the difficulty of differentiating mycobacteria from artefacts [74]. Fluorescence microscopy has been widely used in developed countries but positive slides require restaining with the ZN technique to confirm the presence of acid-fast bacilli. Recently improved fluorescence microscopes using an ultra-bright light-emitting diode have been developed and evaluated by the Foundation for Innovative New Diagnostics (FIND; www.finddiagnostics.org) and are now recommended for implementation [75].

1.2.5 Sputum culture

Sputum samples should be cultured for isolation of bacilli for further identification and drug susceptibility testing wherever possible. Due to the lack of culture laboratories, this is usually only possible at major TB referral centres outside developed countries. Culture of *M.tb* may be performed with solid or liquid culture media. Solid culture generally uses the egg-based Lowenstein-Jensen or Ogawa media, or agar-based Middlebrook 7H10 or 7H11 [76]. Liquid culture techniques include the commercial automated Bactec Mycobacteria Growth Indicator Tube (MGIT) system (Becton Dickinson, USA). *M. tb* produce non-pigmented colonies with a rough and dry appearance ressembling breadcrumbs on egg- based media but on agar-based media, the colonies appear flatter [77] (figure 1.6). The false-positive rate for conventional culture is generally below 5% in a good laboratory, but may occur due to clerical errors or laboratory cross-contamination. Time for results of conventional culture and drug susceptibility testing (DST) are extremely slow on solid media, taking 4-6 weeks for initial isolation and a further 4 weeks for DST. Liquid

culture techniques are faster and more sensitive, and DST may be inoculated directly. Cultures may become positive within one week on the MGIT system, but generally take between 2-3 weeks [78].

Figure 1-6 : Colonies of *Mycobacterium tuberculosis* on Lowenstein Jensen media http://commons.wikimedia.org/wiki/File:M-tuberculosis-on-Lowenstein-Jensen.jpg

During the last two decades, several liquid culture-based methods for cultivation and DST of *M.tb* have been introduced, the most widely used is the MGIT automated system for liquid culture. In this method, a fluorescent quenching-based oxygen sensor is embedded at the bottom of the tube containing an enriched Middlebrook

7H9 broth. The growing mycobacteria consume the dissolved oxygen which generates orange fluorescence when illuminated with an UV lamp. The BACTEC MGIT960 system, that performs incubation and reading of the tubes continuously inside the machine, uses a predefined algorithm to interpret the fluorescent signal and to report the results as positive or negative. The time for detection of *M.tb* from sputum samples in MGIT is about 14 days but the contamination rate can be high (2-10%). The sensitivity and specificity for the BACTEC MGIT960 method are very high 81.5% and 99.6% according to a meta-analysis [78].

The isolation of *M.tb* needs to be confirmed with biochemical tests when performing culture and DST. Characteristics of *M.tb* are slow growth, non-pigmented colonies, positive niacin test. *M.tb* is inhibited by p-nitrobenzoic acid and displays nitratase activity, with susceptibility to pyrazinamide, growth on thiophene carboxylic acid hydrazide (TCH), absence of catalase production at 68°C and absence of iron uptake [76, 77] (table 1.3).

Test	*M.tuberculosis*	*M.Bovis*	*M.bovis BCG*	*M.africanum*	*M.microti*	*M.canettii*
Morphology	rough	rough	rough	rough	rough	smooth
Preferred carbon source	glycerol	pyruvate	pyruvate	glycerol	glycerol	glycerol
Pyrazimidase	+	-	-	+	+	+
Niacin	+	-	-	+/-	+	-
Nitratase	+	-	-	+/-	-	+
Urease	+/-	-	+	+/-	+/-	+
Susceptibility to TCH	R	S	S	S	S	R
O_2 requirement	aerobic	Micro-aerophilic	Micro-aerophilic	Micro-aerophilic	Micro-aerophilic	Unknown

R = resistant, S= susceptible, TCH= Thiophene-2-carboxylic acid hydrazide

Table 1-3: Colony morphological and biochemical characteristics of species in the *Mycobacterium tuberculosis* complex [80]

1.2.6 Drug susceptibility testing

Drug-susceptibility testing (DST) of *M. tb* can be determined by observation of growth or inhibition of growth in a medium containing antituberculous drugs [79]. Conventional methods using egg- or agar-based media are still commonly used and considered the gold standard. In order to reduce the extremely long turnaround times of conventional methodologies, automated liquid culture methods have been developed. Initially, these used radiometric detection as in the BACTEC 460 system but later systems use fluorometric quenching for detection and are therefore safer. As for *M.tb* culture, the most widely used automated system is now the MGIT960 system (Becton Dickinson, USA). The average sensitivities reported by WHO for detection of resistance to INH, RiF, SM, and EMB were 98.7%, 97.2%, 90.8% and 89.3% respectively. The sensitivities to detect susceptibility to INH, RiF, SM and EMB were 98.5%, 96.8%, 93.9% and 94%, respectively [80]. WHO reference standards for the minimum inhibitory concentration (MIC) of antituberculous drugs using the proportion method on LJ medium are 0.2μg/ml -16μg/ml for INH , 4μg/ml for SM, 40μg/ml for RiF and 2μg/ml for EMB [81].

Microscopic Observation Drug Susceptibility (MODS) is a novel technique for the detection of tuberculosis and multidrug-resistant tuberculosis [82]. It is based on three principles: 1) *M.tb* grows faster in liquid medium, 2) *M.tb* with cord formation can be visualized microscopically at an early stage (figure 1.7) and 3) the incorporation of drugs permits the rapid and direct drug-susceptibility testing. The MODS assay uses enriched Middlebrook 7H-9 liquid medium. In the MODS assay, samples are inoculated into drug-free (control) and wells containing the drug of interest. The

MODS assay is a rapid test [82]. MODS sensitivity and specificity for detection of TB in a recent meta-analysis of 21 eligible studies were 96% [95%CI 94-98] and 96% [95%CI 89-100], respectively. The MODS assay is more sensitive than smear microscopy with a median time to detection of 9 days [83]. The sensitivity and specificity for detection of rifampicin resistance pooled estimates are 98.0% [95% CI 94.5-99.3] and 99.4% [95% CI 95.7-99.9%], respectively. Isoniazid resistance is detected with 97.7% [94.4-99.1%] sensitivity and 95.8% [88.1- 98.6%] specificity with a 0.1μg/ml cut-off [84]. However, the cording feature of *M.tb* growth in MODS wells is not sufficiently specific to accurately discriminate *M.tb* from NTM and a recent modification has added P-nitrobenzoic acid (PNB) to one growth well to enable specific identification of *M.tb* [85, 86]. Challenges for this assay are supply of reagents and meticulous technique during inoculation and plate handling to prevent contamination and potential biohazards related to *M.tb* growing in liquid culture [62]. The MODS assay can be used for diagnosis of extrapulmonary tuberculosis with appropriate sample processing [87, 88] .

Other non-commercial phenotypic DST tests include the nitrate reductase assay (NRA) and colorimetric redox indicator (CRI) [89-91]

Figure 1-7 : *M. tuberculosis* characteristic cording in MODS plate at x400 magnification with inverted microscope (courtesy Dr Dang Thi Minh Ha) [92]

1.2.7 Mycobacteriophage assays

Mycobacteriophage based assays using mycobacteriophages to identify *M.tb* from biological specimens requiring only two days to give result and allowing rapid testing for susceptibility to rifampicin showed early promise in small research evaluations but have low specificity in scale up and are not recommended [84, 93]. Mycobacteriophage based assays for *M.tb* are unlikely to be valuable and FIND has now discontinued further development of these assays.

1.2.8 Nucleic acid amplification tests

Nucleic acid amplification tests (NAAT) have been developed in many different formats for diagnosing TB.

Two line probe assays (LPA) are commercially available for detection of resistance to RiF or INH, the INNO-LIPA RIf.TB (Innogenetics, Belgium) and the Genotype MTBDR-*Plus* (Hain Lifesciences, Germany). These assays identify mutations in the *rpoB* gene for RiF resistance and the *katG* gene or *inhA* gene, for INH resistance, through amplification and reverse hybridisation. The advantages of the LPA include relatively short turn-around time ranging from 1 to 2 days and the fact that they can be applied directly on smear positive sputum. A meta-analysis concluded that the MTBDR-*Plus* assay has a high sensitivity (98.1%, [95% CI: 95.9-99.1]) and specificity (98.7%, [95%CI: 97.3 -99.4]) for rifampicin resistance. However, the sensitivity for INH resistance is modest and variable (84.3%, [95% CI :76.6-89.8]) with high specificity (99.5%, [95% CI 97.5%-99.9%]) [94]. Therefore LPA can be used to detect resistance to RIF and INH but cannot be used to rule it out. Following large-scale demonstration projects in high-burden settings, line probe assays were recommended by WHO for use on smear positive sputum but are not recommended as a complete replacement for conventional culture and drug-susceptibility testing [95].

The Xpert MTB/RIF test (Cepheid, USA), endorsed by the WHO in 2010, is an automated molecular test for detection of *M.tb* and resistance to RiF (figure 1.8). A Cochrane review of 15 studies with a total of 7,517 participants concluded Xpert has a pooled sensitivity of 88% (95% CI 83% to 92%) and pooled specificity of 98% (95% CI 97% to 99%) when used as an initial test replacing smear microscopy [96, 97]. Compared with phenotypic drug-susceptibility testing, for rifampicin resistance detection (11 studies, 2340 participants), Xpert achieved a pooled sensitivity of 94% (95% CI 87% to 97%) and pooled specificity of 98% (95% CI 97% to 99%). In TB

patients with HIV co-infection, the sensitivity of smear microscopy for the diagnosis of TB is very low but GeneXpert sensitivity is relatively unaffected by HIV co-infection, so the use of XpertMTB/RIF has the potential to increase the detection of TB among HIV-infected individuals. The cost of the test is still high compared to smear microscopy but less costly than culture and drug-susceptibility testing. However, FIND has negotiated cost reductions for use in low-and middle- income countries with the cost for the GeneXpert 4-module instrument of US $17,500 and the cost per cartridge of $10 [98]. The test gives a result in 2 hours from inoculation of the cartridge [97].

Figure 1-8 : Assay procedure for the MTB/RIF test

Reproduced from:

http://www.finddiagnostics.org/export/sites/default/programs/tb/images/xpert/xpert_mtb-rif_procedure.JPG

1.2.9 Sputum induction

Apart from sputum examination using microscopy and culture to diagnose pulmonary TB, sputum induction can be used to improve sample collection and test sensitivity. According to a meta-analysis study from Hepple *et al.*, the overall success of sputum induction was high from 76.4% (95% CI 68.5-83.2) to 100% (95% CI 98.5-100) and side effects were mild and rare. The researchers concluded that this technique increases TB case detection, and should be used for patients who cannot expectorate spontaneously, or whose sputums are negative on smear [99]. However, sputum induction carries increased risk to the clinical staff and other patients and appropriate precautions should be implemented.

1.2.10 Immunological tests

A method that has been used widely for diagnosis of tuberculous infection is the tuberculin skin test (TST). This test is not effective in diagnosis of active TB disease rather than infection, particularly in low- and middle-income countries where there is a high prevalence of *M.tb* infection and the BCG vaccination is frequently administered as the TST is confounded by prior BCG vaccination. Some countries, such as the USA, have historically not used universal BCG vaccination in order to preserve the diagnostic utility of TST. TST uses tuberculin that is obtained from a sterilized and concentrated *M. tb* culture filtrate. In 1934, Seibert prepared a purified protein derivative (PPD) called tuberculin [100]. In 1951, PPD was adopted and used

by the WHO [101]. Nowadays, PPD-RT23 is most widely used worldwide. Tuberculin should be stored at 4°C to 8°C.

The tuberculin test involves the administration of tuberculin via the intradermal route (this test is called intradermal reaction: IDR) (figure 1.9). When tuberculin penetrates the skin, phagocytosis of the material produces an inflammatory reaction. In non-sensitised subjects, this reaction disappears rapidly, however in subjects with sensitization of prior mycobacterial infection, this reaction intensifies. The T lymphocytes that were previously sensitized with *M.tb* antigen secrete lymphokines generating lymphomonocytic perivascular infiltration. The host response to the tuberculin starts within 5 to 6 hours, peaking after 48 to 72 hours and persisting for several days. This phenomenon represents a cell-mediated delayed immune hypersensitivity reaction. The reading of result should be obtained 48 to 72 hours after performing the test by measuring along the transverse diameter of induration in millimetres. The interpretation of positive or negative results of TST depends on different clinical situations of patients, including BCG vaccination status, HIV status and age. It is therefore relatively subjective in interpretation [101].

Following *M.tb* infection, between 2 and 12 weeks are required for the sensitized T Lymphocytes to be able to recognize tuberculin. False-positive tuberculin test can be due to multiple reasons. The most important is infection involving environmental mycobacteria or due to BCG vaccination. Alternative PPD preparations are available to assist in the differentiation of NTM infection but are not widely used [102, 103].

Figure 1-9 : Photograph showing administration of the tuberculin skin test. Reproduced from: A tuberculosis guide for specialist physicians [104].

Interferon-γ release assays (IGRA) measure Interferon-gamma (IFN-γ) response to *M.tb* antigens. ESAT-6 and CFP-10 are specific to *M.tb* whereas PPD will be confounded by BCG vaccination. There are two major commercial IGRA tests available, QantiFERON-Gold (QFT-G, Qiagen, Netherlands) which measures the concentration of interferon- γ using ELISA, and T-SPOT.TB (Oxford Immunotec, UK) which counts the number of cells that release interferon-γ manifested as spots. The QFT-G test is available as an "in-tube" version (QFT-G-IT) which includes antigen TB7.7 together with ESAT-6 and CFP-10 [105]. The specificity of IGRA is high and thought to be superior to that of TST whereas the sensitivity is variable between studies. It is extremely difficult to evaluate the specificity of IGRAs as no suitable gold standard exists and long-term cohort follow-up studies are costly. It has

been difficult to establish cut-off values for differentiating between active and latent infection and in 2011 WHO issued a negative advisory that IGRA should not be used for the diagnosis of active TB in high-burden settings [105].

IGRAs including QFT and T-SPOT are considered to be more accurate tests compared to TST for latent TB and both are widely used for identifying recent contacts with LTBI in low-burden settings [106].

Other commercial serodiagnostic tests for TB currently marketed are not accurate and WHO issued an unprecedented negative advisory against the use of such tests in 2010 following extensive evaluation [107].

1.2.11 Biomarkers

Many biomarkers have been studied in TB infection and disease but only two biomarkers- C-reactive protein (CRP) and procalcitonin (PCT) are of clinical value. CRP is an acute phase protein produced by the liver in response to inflammation that binds to phosphocholine on the surface of dead or dying cells to promote phagocytosis. Serum CRP levels increase in the advanced stages of TB and decrease with treatment. PCT is a peptide precursor of the hormone calcitonin that is involved in calcium homeostasis. PCT increases in bacterial inflammation and is not elevated in pulmonary TB so it can be used to differentiate between TB and pneumonia [108]. Until now, accurate rapid point-of-care diagnostic tests have not been developed and novel biomarker discovery remains challenging. Many promising biomarkers have been investigated but more trials according to international research standards need to be conducted [109].

1.2.12 Radiological imaging

Chest X-ray (CXR) is the most useful tool for diagnosis of lung infections but is highly dependent on the skill and experience of the reader. Although certain features such as hilar lymphadenopathy can be highly suggestive of TB, CXR can neither confirm nor rule-out a diagnosis of TB [110].

In primary TB, infiltrates or consolidations visible on CXR may affect any lung segment but the middle and lower lobes are characteristic of TB. Consolidations are generally dense and homogenous in appearance. The most characteristic radiological feature in primary TB is image of lymphadenopathy. Lymph node involvement is generally unilateral with the hilar and paratracheal regions most often affected [110].

In post-primary TB, chest radiography often reveals patchy infiltrates or poorly defined segmental consolidation. These pulmonary abnormalities are usually located in more than one segment or lobe and the apical and posterior segments of the upper lobes, or the superior segment of the lower lobe are most commonly affected. Single or multiple cavitations with thick walls and irregular margins are the hallmark of this form. There may be a small quantity of fluid in the cavitation manifesting as an air-fluid level. Nodular opacities are another common feature. Only approximately five percent of patients will have a tuberculoma visible on CXR.

Computerized Tomography Scan (CT scan) of the lung can be used to enhance detection of TB in patients with a normal or inconclusive CXR. Chest CT scan is useful to describe lesions and their positions. Moreover, CT scan can detect complications and lesions in pleura and mediastinum [110].

In primary TB, chest CT is consistently shown to be more sensitive than plain CXR films for the detection of lung lesions and enlarged lymph nodes. In post-primary TB, radiologic findings in CT are the "tree-in-bud" (nodular opacities), centrilobular small nodules, patchy or lobular areas of consolidation and cavitation. In miliary TB, the 'scattered millet' appearance is seen from numerous nodules scattered throughout both lungs 1-3 mm in diameter. Generally, differences between drug sensitive and drug resistant TB on radiological imaging can be attributed to differences in disease progression.

Recently, positron emission tomography has been investigated as a non-invasive method to monitor disease activity and responses to antituberculosis therapy but this is not feasible on a large-scale and is likely to be limited to monitoring disease progression in intractable cases [111, 112].

1.2.13 Bronchoscopy

Fiberoptic bronchoscopy is used in suspected TB cases which are sputum smear negative to obtain bronchial washing/aspirate samples to confirm the presence of mycobacteria or obtain a differential diagnosis. Bronchial brushing and bronchoalveolar lavage fluid, in postbronchoscopy sputum and biopsy samples may also be examined by smear, culture and nucleic acid amplification for mycobacteria. The most common bronchoscopic findings of TB are congestion, swelling, hyperaemia, whitish plaques of variable size, ulceration, erosion and granulation. The segmental opening may be narrowed and slightly deformed [113].

1.2.14 Genotyping of *M.tb*

Recently, progress has been made in understanding the basic biology of *M.tb*. The field of mycobacterial genetics, the development of the field of molecular epidemiology and the sequencing of the entire *M.tb* genome have been key factors. IS6110 restriction fragment length polymorphism (RFLP) typing allowed investigators to analyze clinical isolates to study transmission of tuberculosis in communities, leading to a clearer understanding of TB transmission [114]. In 1998, the complete DNA sequence of *M.tb* (strain H37RV) was reported, which raised hopes of insights into disease pathogenesis [115]. Comparative genomics of mycobacteria have made it possible to construct evolutionary trees which elucidate the phylogenetic evolution of human disease [4, 116]. In addition, genomics has been used to compare virulent to avirulent *M.tb* strains, as well as immunogenic from nonimmunogenic strains. These insights from genomics may help to develop effective tuberculous vaccines in the future since the immune correlates of protection against TB remain unknown [117].

M.tb from different lineages may have different clinical phenotypic properties but the evidence is often conflicting and so the correlation of genotype and phenotype has remained elusive [118]. In one study from Gambia, researchers found that transmission of *M.tb* to contacts was similar among *M.tb* strains from different lineages. However, progression to active TB within a 2-year follow-up period varied by lineage; 5.6% of those exposed to East-Asian lineage, 1.2%-3.9% exposed to Euro-American lineage and only 1% of people exposed to West African lineages [119].

IS*6110* Restriction Fragment Length Polymorphism (IS*6110* RFLP) is a genotyping technique for *M.tb* which can be used to obtain "fingerprints" for different isolates of

M.tb dependent upon the location and number of IS*6110* insertions. The mycobacterial insertion sequence IS*6110* has been shown to be present in multiple copies in the chromosome of some strains of *M.tb*. In RFLP analysis the *M.tb* chromosomal DNA is digested by a restriction enzyme and the resulting DNA fragments are separated according to their length by gel electrophoresis. The insertion sites of IS*6110* are then detected by hybridization to an *IS 6110* specific probe.

IS6110 RFLP fingerprinting of *M.tb* was the standard identification method in studies on transmission of tuberculosis in the 1990's [120]. It has now been largely superseded by other, simpler techniques.

Variable-Number Tandem Repeat-Mycobacterium Interspersed Repetitive Units (VNTR-MIRU) is the *M.tb* specific name of a multiple locus VNTR analysis-based typing scheme. MIRU VNTR has become an alternative method as it allows high-throughput, discriminatory and reproducible analysis of clinical isolates resulting in a more reliable and faster method for transmission analysis [121, 122].

Spoligotyping is based on polymorphisms in the "Direct Repeat" (DR) region, of *Mycobacterium tuberculosis* complex bacteria [123, 124]. The DR region consists of a non-variable repeated sequence of 36 base-pairs length which is interspersed with variable spacer regions, each of which are between 35 to 41 base pairs in length. One DR and its neighbouring non-repetitive spacer is called " Direct Variant Repeat" (DVR). Deleted sequences of DNA within the DR region result in variations in the presence or absence of the variable spacers between strains of *M.tb*. The presence of spacers is detected by PCR amplification targeted to the non-variable repeat sequence. The resulting amplicons are then hybridised to probes specific for each variable

spacer sequence on a membrane and the presence or absence of spacers recorded as a binary code. The short sequence amplification required for spoligotyping make it a relatively robust technique which can be applied directly to smear positive clinical samples. Many studies have evaluated the clinical usefulness of spoligotyping for simultaneous detection and typing of *M.tb* strains [125]. Spoligotyping can be a useful method for rapid outbreak identification in low incidence such as in nosocomial outbreaks or in the management of transmission of multidrug-resistant tuberculosis [126, 127].

Large sequence polymorphisms (LSPs) can define the six principal lineages of the *M.tb* complex including Euro-American, East-Asian, Indo-Oceanic, East-African-Indian (EAI) and the West-African lineages 1 and 2. The robustness of LSPs has been validated by multilocus DNA sequence analysis. Spoligotype families are sub-lineages within the main lineages defined by LSPs [128].

1.2.15 Treatment

There are believed to be four hypothetical populations of *M.tb* that may exist in patients with TB including actively growing organisms, slow intermittently growing organisms, organism surviving under microanaerobic conditions in a low pH environment, and completely dormant organisms surviving under anaerobic conditions. There are also three mechanisms of major activities of anti-tuberculosis drugs: (1) Drugs which have ability to kill actively growing bacilli rapidly such as INH, RiF and SM (bactericidal); (2) Drugs with the ability to kill the semi-dormant organisms such as RiF and PZA (sterilizing); and (3) Drugs which have ability to

prevent the emergence of bacillary resistance including INH, RiF, SM, EMB and PZA (bacteriostatic).

The goals for TB treatment are to cure the individual patient and to minimize the transmission of *M.tb* to other persons. The prinicipal recommended regimen for drug sensitive TB in newly diagnosed patients is a two month 'intensive phase' of treatment with INH, RiF, EMB and PZA followed by a four month 'continuation phase' of RiF and INH (2HRZE/4HR) [129]. Alternative treatment regimens are recommended in cases of known or suspected drug resistance, severe forms of TB and other special situations. Although no longer recommended by WHO, several NTPs still use an eight month treatment regimen (2HRZE/6HE or 2HRSZ/6HE). The 6-month regimen (2RHZE/4RH) was significantly superior to the 8-month regimen (2RHZE/6HE) in a multi-country RCT conducted by the IUATLD RCT . A 7-month continuation phase regimen is recommended for patients with cavitary pulmonary TB, positive sputum culture at completion of initial phase, and lack of PZA in the initial phase. In populations with known or suspected high levels of bacillary resistance to INH, WHO has suggested that RiF, INH and EMB should be used rather than RiF and INH in the continuation phase, but there is no evidence for the efficacy of this regimen and the recommendation is based on expert opinion only [129]. The twice-weekly regimens should not be used because of the risk of treatment failure when missed doses occur. For treatment of relapse cases with smear positivity or retreatment after interruption, the 8-month regimen of $2SRHZE/RHZE/5R_3H_3E_3$ is recommended. However, this regimen is controversial because it 'adds a single drug

to a failing regimen' and has been shown in several studies to have poor efficacy [129].

In 1993, WHO announced the DOTS (directly observed treatment short course) strategy comprising 5 key components consisting of (1) a network of trained health-care or community workers to administer DOT, (2) properly equipped laboratories with trained personnel to perform sputum microscopy for TB diagnosis , (3) a reliable supply of high-quality drugs, (4) an accurate record keeping and cohort analysis system for monitoring case-findings and outcomes and (5) sustained political commitment and funding [130].

The bactericidal and sterilizing activities of rifampicin as well as their dose and concentration dependence have been demonstrated in many studies [131]. Higher doses than the 600mg standard TB dose of rifampicin have been studied for diseases including brucellosis without detection of significant increase in severe adverse events and many now advocate that a higher standard dose should be trialled for pulmonary TB [132]. Mild hepatotoxicity occured more frequently among patients who received high-dose rifampicin in one study although no patients developed serious hepatotoxicity [133]. The safety and tolerability of high-dose rifampicin needs to be assessed meticulously. A systematic review showed high dose of RiF resulted in improvement of culture conversion rates. However, side effects such as flu-like syndrome were seen when using RiF intermittently [134]. A phase II clinical trial is planned but not yet recruiting to compare the pharmacokinetics and pharmacodynamics of daily doses of 1200 mg and 900 mg of rifampicin with the standard dose (600mg) during the intensive phase of treatment with a view to the

potential of high dose RiF to shorten TB treatment (ClinicalTrials.gov Identifier : NCT01408914) [135].

It is recommended that all patients with tuberculosis should be given counselling for HIV and tested due to the high susceptibility of HIV-infected individuals to TB disease [67]. Patients also should be tested for hepatitis B and C virus infection using serological tests if they have risk factors such as injecting drug use, birth in Asia or Africa, and HIV infection. Routine blood tests are also recommended to establish liver function and the risk of adverse drug reactions such as baseline measurements of serum amino transferases (aspartate aminotransferase AST, alaninne aminotransferase ALT), bilirubin, alkaline phosphatase, serum creatinine and a platelet count. Testing of visual acuity and red-green color discrimination should be obtained when EMB is to be used as a baseline for ocular toxicity monitoring.

During pulmonary TB treatment, ideally a sputum specimen for microscopic examination and culture should be checked at a minimum of monthly intervals until two consecutive specimens are negative on culture [136]. In practice, monthly culture is not feasible in high-burden settings and most TB patients never receive a culture test. Treatment is monitored via sputum smear microscopy alone. Patients should be clinically evaluated at least monthly to identify any adverse effects of the anti-tuberculosis medications and to assess adherence.

Bacteria may continue to be expectorated several months into treatment and dead bacilli will give positive smear results. Treatment failure is defined by WHO as a positive *M.tb* culture or AFB smear after at least 5 months of treatment (table 1.4). In high-burden settings due to the lack of resources for culture, most failure cases are

identified by smear positivity at 5 months of treatment or later. Relapse is defined as recurrent tuberculosis at any time after completion of treatment with apparent cure at treatment completion of the previous episode of TB. Cases classified as relapse may be cases of reinfection as the necessary genotyping to define relapse is normally only performed in a research context.

[a] These definitions apply to pulmonary smear-positive and smear-negative patients, and to patients with extrapulmonary disease. Outcomes in these patients need to be evaluated separately.

[b] The sputum examination may not have been done or the results may not be available.

[c] For smear- or culture-positive patients only

Table 1-4: Definitions of treatment outcomes

From: Treatment of tuberculosis, 4th Edition,WHO (2009) [129]

The use of anti-TB drugs in fixed-dose combination (FDC) tablets comprising 2, 3 or even 4 drugs can be convenient for prescription and adherence. The use of FDC reduces medication error, simplifies drug procurement and supply, and decreases the risk of development of drug resistance and increases treatment adherence. However, FDC drugs may still be taken irregularly and so the need for treatment supervision remains. Many studies have shown that there are no significant differences between FDC and single tablets regarding treatment outcome using measures such as sputum conversion rate and relapse, and no increased incidence of adverse events [137]. The major multicountries trial conducted by the IUATLD and known as 'study C' published in 2011, showed noninferiority of FDC and that use of FDC had potential advantages compared to a regimen of separately given drugs [138]. In contrast, one study, conducted in Singapore, found a higher relapse rate at 2 and 5 years after treatment completion in patients who received FDC tablets compared with those who received single tablets [139]. Patients were followed for 18 months and it is therefore possible performace of FDC regimens was overestimated due to occurrence of undetected late relapse.

Possible reasons for treatment failure include non-adherence, drug resistance and malabsorption of anti-TB drugs. Social exclusion including homelessness, alcohol or substance abuse, behavioural problems, mental retardation and lack of social or family support are risk factors for non-adherence to the TB treatment [140].

1.2.16 Surgery

Adjunctive surgery for pulmonary TB improves the chance of cure in some patients with drug-resistance. Three basic criteria for surgery are anti-TB drug resistance with a high probability of failure or relapse with medical therapy only, localized TB lesions, and after lung resection sufficient drug activity for TB medical therapy. Chemotherapy should be given 3 months before surgery [112].

1.2.17 Adjunctive therapies

In case of severe respiratory insufficiency, central nervous system and pericardial involvement, corticosteroids can be used to modulate the immune response which is thought to be responsible for much of the tissue damage and ensuing complications [141-143]. In cases of tuberculous meningitis, adjunctive corticosteroids are recommended in all cases as meta-analysis of available trial data has demonstrated their efficacy in reducing mortality and neurological sequelae [144]. There is insuffient data to prove a beneficial effect of steroids in cases of HIV-associated TBM and no trials establishing optimum formulation, dosage and duration. A recent systematic review suggested a mortality benefit for steroids across all forms of TB, including pulmonary, but more data is required from high quality randomized controlled trials to confirm this [145].

Immunotherapy such as cytokine supplementation and *Mycobacterium vaccae* have been tried and some approaches have shown moderate benefit in cavity resolution and sputum sterilization but have not been sufficiently effective to be adopted as routine measures [146-148]. In early studies Vitamin D showed potential in TB treatment by

enhancing mycobacterial killing by macophages but clinical trial data has shown conflicting results of vitamin D supplementation on outcomes [149-151].

1.2.18 Paradoxical response

Paradoxical reaction is a condition in which patients have a temporary exacerbation of symptoms, signs and radiographic manifestations of TB after TB treatment and is particularly common in cases of HIV co-infection, when it is known as immune reconstitution inflammatory syndrome (IRIS) [152]. Manifestations may include high fever, new or exacerbated lymphadenopathy, central nervous system lesions, expanded pulmonary parenchymal infiltration, and pleural effusion. Other differentials should be ruled out such as treatment failure before diagnosis of paradoxical reaction. Changes in TB treatment regimen are questioned in paradoxical reaction. In cases which the clinical situation is severe, corticosteroids should be used at a dose of about 1mg/kg and gradually reduced after 1 to 2 weeks [153].

1.2.19 Treatment for special situations

Renal insufficiency and end-stage renal disease: RiF and INH are metabolized by the liver so the dosing of these two drugs is not changed. PZA is also metabolized by the liver but its metabolites may accumulate with renal insufficiency. EMB is 80% cleared by the kidney so it is accumulated in renal failure. A longer interval between doses of PZA and EMB is recommended, for example, three times a week. In patients

on hemodialysis, drugs should be given after this procedure to avoid removal of the drugs [129].

Hepatic disease: regimens with fewer potentially hepatotoxic agents should be recommended, such as regimens without INH, regimens without PZA, regimens with only one potentially hepatotoxic drug, and regimens with no potentially hepatotoxic drugs at all [129].

Pregnancy and breastfeeding: Streptomycin is contraindicated in pregnancy but RiF, INH and EMB may be used. There is insufficient data on the use of PZA during pregnancy and the US guidelines do not recommend its use, while WHO and IUATLD do. The degree of advancement of disease should be taken into account when deciding to initiate treatment. There is no evidence that breastfeeding while taking first line TB medications is harmful to the infant [129].

Tuberculosis and diabetes mellitus: a link between tuberculosis and diabetes has been suspected for a long time. The dramatic increases in adult diabetes incidence in the last few decades have focused attention on the link between diabetes and susceptibility to TB [154]. In addition, TB may exacerbate glycaemic control dysfunction in diabetic individuals [155, 156]. Diabetic patients, who need more than 40 units of insulin per day, or whose haemoglobin A1c concentration is greater than 7%, are more likely to develop tuberculosis according to a cohort study of over 40,000 individuals from Hong Kong [157]. Diabetes may also influence the radiographic clinical presentation of TB, a large proportion of diabetic patients with tuberculosis had lower-lung involvement, multilobar infiltration and multiple cavities in one series [158]. Impaired immunity in diabetic patients may lead to increased

bacterial burden, slower bacterial clearance on treatment and higher rates of failure and relapse. Diabetes can alter oral absorption, decrease protein binding of drugs and lead to renal insufficiency which can substantially alter the pharmacokinetics of antituberculosis drugs, which may be partially responsible for the increased treatment failure. For example, in a small study from Indonesia, diabetic TB patients had rifampicin serum concentrations 53% lower than those of non-diabetic TB patients [159]. In treatment of TB patients with diabetes, therapeutic drug monitoring has been suggested but is not currently practical outside a research setting [155].

Tuberculosis and tobacco: Smoking is a major risk factor for tuberculosis. Passive exposure to tobacco and other forms of smoke also carries an increased risk [160]. Smokers are more likely to have cough, dyspnoea, appearances of lesions on upper zones on chest radiography, cavity and miliary appearance, and positive sputum culture, probably as a consequence of the pre-existing lung-damage [161]. In studies investigating the influence of smoking on time to sputum culture conversion, findings have been conflicting and may be confounded by the level and duration of smoking history (pack-years). Exposure to tobacco smoke influences treatment outcome. Smoking has been significantly associated with default and failure of treatment, but was not with death, compared to non-smokers. A case-control study of 111 cases and 333 controls from India showed an association between tobacco smoking and pulmonary tuberculosis with OR of 3.8 [95% CI 2.0-7.0] [162]. TB Patients need and should receive counselling and assistance for smoking cessation and these interventions are becoming more widely promoted in high-TB incidence countries, but are still extremely limited [162].

1.2.20 Complications of TB

Without treatment of TB, the disease will usually progress and eventually result in death. In addition, TB disease can spread to other parts of the body through bacteriaemic seeding. The most common acute complications are lung damage, adult respiratory distress syndrome, pleural effusion, pneumothorax, empyema, bronchiectasis, haemoptysis, TB meningitis, miliary TB, joint and bone TB, TB peritonitis, TB pericarditis, TB lymphadenitis, bowel TB, fallopian TB and tuberculoma. Immune reconstitution inflammatory syndrome (IRIS) is a common but poorly defined complication of HIV-associated TB once ARV treatment is initiated. The late complications are aspergilloma, bronchogenic carcinoma, tracheobronchial stenosis and bronchiectasis. TB can cause vascular lesions including pulmonary and bronchial arteritis, thrombosis, bronchial artery dilatation and Rasmussen aneurysm. Mediastinal lesions such as lymph node calcification, oesophago-mediastinal fistula and oesophago-bronchial fistula are also seen in TB complications. A rare complication of pulmonary TB is deep vein thrombosis (DVT) [163].

1.3 Drug-Resistant Pulmonary Tuberculosis

1.3.1 Epidemiology

Drug resistant strains of tuberculosis are increasing in prevalence globally and represent one of the major threats to TB eradication due to the lack of novel second-line agents [164]. Although drug resistance was identified immediately following the

clinical application of streptomycin, the early implementation of multi-drug therapy for TB has actually preserved the limited number of truly effective first-line drugs remarkably well. However, multiple new drug classes are now urgently needed to allow effective eradication of drug-resistant forms of TB.

In 2008, an estimated 3.6% of all TB cases were multidrug resistant TB (MDR-TB, resistant to at least INH and RiF), numbering between 390,000 – 510,000 cases wordwide, of which 150,000 resulted in death [164]. Fifty percent of MDR-TB cases occurred in China and India. Twelve countries have reported proportions of MDR-TB of 6% or more among new TB cases and five of these countries reported rates of 50% or more among previously treated cases. Extensively drug-resistant TB (XDR-TB) was defined by WHO in 2006 as MDR *M.tb* additionally resistant to a fluoroquiniolone and an injectable second-line agent. Worldwide, 5.4% of MDR-TB cases were estimated to be extensively drug resistant TB (XDR-TB). By 2010, 58 countries had reported at least one case of XDR-TB [164].

The total number of estimated MDR-TB cases in Vietnam ranks the third highest in the Western Pacific region. There were an estimated 4,047 new cases (95% CI, 2341 to 6056) and 2,374 previously treated cases (95% CI, 1378 to 3535) of MDR-TB (www.who.int/tb/data, September 2013). The prevalence of MDR-TB was 2.7% (95%CI, 2% to 3.6%) in new TB cases and 19.3% (95CI, 14.2% to 25.4%) in retreatment cases. Vietnam has developed the programmatic management of drug-resistant TB (PMDT) from 2009 in five cities and provinces including Ho Chi Minh City and Hanoi [165]. FQs are widely used for treating respiratory infections,

therefore the prevalence of FQs resistant *M.tb* is substantial in some regions, but is not yet high in Vietnam according to the limited available data.

1.3.2 Classification and definition

TB is classified as Drug- resistant TB (DR-TB) if the infecting isolate of *M.tb* grows *in vitro* in the presence of one or more antituberculosis drugs. Some general terms for drug resistant bacteria have unique and specific definitions in the context of drug resistant TB and are listed below for clarity.

Mono-resistant TB is resistant to a single antituberculous drug.

Poly-resistant TB is resistant to more than one antituberculous drug, but not resistant to both isoniazid (INH) and rifampicin (RiF).

Multi-drug resistant TB (MDR TB) is resistant to at least RiF and INH.

Extensively drug-resistant TB (XDR TB) is defined as MDR-TB with additional resistance to a fluoroquinolone (FQs) and at least one of second-line injectable drugs (kanamycin (Ka), amikacin (Am) or capreomycin (Cm)).

Totally drug-resistant TB (TDR) is not recognized as a definition by the WHO but has been used to describe strains that are resistant to all first and second-line drug classes (for example, aminoglycosides, cyclic polypeptides, fluoroquiolones, thioamides, serine analogues and salicylic acid derivates). Lack of standardization of laboratory tests for resistance to second-line agents make reports of TDR TB unreliable. The control, prevention and treatment of such deadly bacilli challenges TB programmes worldwide [166].

1.3.3 Mechanism of drug resistance in *M.tb*

After the introduction of anti-TB drugs, *M.tb* strains with resistance may be selected. Resistance in *M.tb* is due to spontaneous chromosome mutations at a frequency of between 10^6 to 10^8 replications, depending on the drug, and therefore resistant organisms will be present in the absence of drug pressure. *M.tb* does not acquire resistance through DNA transfer or plasmid acquisition [167]. In theory, a drug resistant population will not emerge if at least three effective drugs are used. Irregular drug supply, inappropriate prescription and poor adherence are errors leading to clinically drug-resistant TB. The transmission of resistant *M.tb* strains from an index patient to others aggravates the problem.

1.3.3.1 Isoniazid resistance mechanism

INH has the highest early bactericidal activity (EBA) of all antituberculous drugs, however it is only active against growing tubercle bacilli, but not active against non-replicating or anaerobic tubercle bacilli. INH is a prodrug which is activated by the catalase-peroxidase enzyme (katG) encoded by the *katG* gene [168]. The primary target of INH inhibition is the *inhA* enzyme (enoyl-acyl carrier protein reductase) involved in elongation of fatty acids in mycolic acid synthesis [169]. Recent studies showed that INH-NAD(P) adducts react with other protein targets besides *inhA* such as DfrA (an NADPH-dependent dihydrofolate reductase involved in DNA synthesis) [170]. The MIC of INH is 0.02-0.2 μg/ml. *In vitro*, resistance to INH occurs at a frequency of 1 in 10^{5-6} bacilli. INH-resistant clinical isolates of *M.tb* may lose the catalase peroxidase enzyme, conferring complete resistance due to the need for the *katG* gene product to activate the pro-drug, but at considerable fitness cost [171].

KatG S315T is the most common INH resistance mutation. The prevalence of the mutation among INH resistant strains varies between 50-95% geographically for unknown reasons [172]. Mutations at other sites in *KatG* can also confer resistance to INH. Mutation at codon 463 was initially thought to be resistance associated, but has now been shown to be a phylogenetic mutation [173, 174]. Resistance to INH also commonly occurs by mutation in the promoter region of *mabA/inhA* or, more rarely, by mutations at the *inhA* active site, lowering the InhA affinity to the INH-NAD adduct (low-level resistance MICs=0.2-1μg /ml) . Mutation in *inhA* can confer cross-resistance to ethionamide (ETH) [169]. Ninety percent of INH- resistant isolates in Vietnam have at least one mutation in the *katG* or *inhA* promoter gene regions [175]. Mutation in the promoter region of *ahpC*, encoding an alkylhydroperoxide reductase, increases expression of the enzyme and compensates for the lack of catalase-peroxidase. About 10-25% of low level INH-resistant strains have no known resistance mutations. Mutation in *mshA* encoding an enzyme of mycothiol biosynthesis has shown INH and ETH resistance *in vitro* [176].

Mutations in *katG* confer higher levels of resistance, generally >5μg/ml, than mutations in the *inhA* promoter or *aphC* genes (1-2μg/ml, or ≥5 times the MIC). [3, 177, 178]. Mutation in the inhA promoter region, usually C-15T, confers low level INH resistance due to overexpression of *inhA*. In addition, mutations in the *inhA* structural gene result in an increased dissociation constant for the inhA-NADH-complex [179, 180]. Mutation in *inhA* can confer cross-resistance to ethionamide (ETH) [169]. Mutation in *mshA* encoding an enzyme of mycothiol biosynthesis has also shown INH and ETH resistance *in vitro* [176].

The role of ahpC (alkyl hydroperoxidase reductase) is to detoxify organic peroxides and mutations are thought to lead to overexpression which compensates for the loss of catalase peroxidase activity [181, 182].

Mutations with changes in the genes, *kasA* (ß-ketoacyl ACP synthase) and *ndh* (NADH dehydrogenase) have also been observed in INH resistant isolates but a meta-analysis failed to confirm a role in clinical INH resistance [172, 183-185].

M.tb, in common with other bacteria, uses efflux pumps to expel harmful substances from their cytoplasm. INH efflux is thought to occur via a reserpine-inhibitable pump encoded by a three-gene *M.tb* operon, *iniABC* [186], but other as yet uncharacterised mechanisms are also likely to exist since 10-20% of phenotypically INH resistant isolates have no known mechanism of resistance. Recently, some new mutations potentially linked to INH resistance have been described in mabA, Thr4Ile, from 5 patients with different strains whose sputum cultures remained postitive after standard TB treatment [187]. The identification of efflux pumps may lead to a new way to enhance antituberculosis therapy, through inhibition of this mechanism [188] and it is likely that whole genome sequencing of resistant isolates will give further insights into INH resistance mechanisms in the next few years [189-191].

1.3.3.2 Rifampicin resistance mechanism

RiF is a bactericidal antibiotic that is active against both growing and stationary phase *M.tb* bacilli. Strains with MICs <1 µg/ml in liquid or agar medium or MICs < 40 µg/ml in Lowenstein-Jensen medium are considered RiF-susceptible. RiF interferes with RNA synthesis by binding to the β subunit of the RNA polymerase at the catalytic center resulting in blockage of the elongation of the RNA chain.

Spontaneous resistance to RiF occurs at a frequency of 1 in 10^7 to 10^8 bacilli via mutations in a defined 81 base pair (bp) region of the *rpoB* gene. Mutations at codons 531, 526 and 516, occur in 96% of RiF-resistant *M.tb* [192] and generally result in high level resistance (MIC > 32μg/ml) and cross-resistance to all rifamycins. Specific mutations in codons 511, 516, 518 and 522 are related to lower-level resistance to RiF and rifapentine but susceptibility to rifabutin and rifalazil [193].

1.3.3.3 Pyrazinamide resistance mechanism

PZA is an important drug because it kills persistent bacilli in acidic pH environments and plays a crucial role in shortening TB treatment [194]. PZA is not active against *M.bovis* [195]. The MIC ranges from 6.25-50 μg/ml. PZA is a prodrug that requires conversion to its active form pyrazinoic acid (POA) by the pyrazinamidase/nicotinamidase enzyme encoded by the *pncA* gene of *M.tb* [196]. The target of PZA is related to membrane energy metabolism [197]. POA reaches the cell surface through passive diffusion and a defective efflux. The acid pH facilitates the formation of uncharged protonated POA and PZA can permeate through the membrane to accumulate and disrupt the membrane of *M.tb*. PZA-resistant *M.tb* strains with *pncA* mutations, lose pyrazinamidase/nicotinamidase activity [198]. Resistance testing to PZA *in vitro* is unreliable due to the need for an acid environment and reports of the prevalence of *pncA* mutations among resistant strains vary widely [199, 200]. It is unclear if this is due to variations in the phenotypic DST accuracy or due to other factors, but it is apparent that other undiscovered or poorly characterized mechanisms of resistance exist [201].

1.3.3.4 Ethambutol resistance mechanism

EMB is a bacteriostatic agent that is active for growing bacilli with MICs of 0.5-2 μg/ml. EMB interferes with the biosynthesis of arabinogalactan in the cell wall via arabinosyl transferase, an enzyme encoded by *embB* [202]. Strains resistant to EMB have MICs > 7.5 μg/ml. *In vitro,* spontaneous EMB resistance occurs at a frequency of 1 in 10^5 bacilli. The most frequently occurring EMB resistance mutation is at codon 306 of the *embB* gene. Approximately 65% of EMB-resistant strains have *embB* mutations [203].

1.3.3.5 Mechanism of aminoglycoside resistance

SM is an aminoglycoside antibiotic that is active against various bacterial species including actively growing *M.tb* with MICs of 2-4 μg/ml. SM inhibits protein synthesis by binding to the 30S subunit of ribosomes causing misreading of the mRNA message during translation. The site of action of SM is the 30S subunit of the ribosome at the ribosomal protein S12 and the 16S rRNA [204]. Mutations in the S12 protein are encoded by *rpsL* gene and 16S rRNA encoded by *rrs* gene [205]. A mutation in *gidB* encoding a conserved 7-methylguanosine methyltransferase specific for 16S rRNA causes low level SM resistance [206].

The second-line tuberculosis drugs, kanamycin (KM) and amikacin (AMK), also belong to the aminoglycoside class and inhibit protein synthesis through modification of ribosomal structures at the 16S rRNA [207]. Mutations at 16S rRNA (rrs) position 1400 are related to high-level resistance to KM and AMK [208]. Capreomycin (CPM) is a polypeptide antibiotic. A gene called *tlyA* encoding rRNA methyltransferase is involved in resistance to CPM [209]. Cross-resistance can occur between KM, AMK

and CPM and depends on the resistance mechanism of an individual strain. SM-resistant strains are often susceptible to KM and AMK [210, 211].

1.3.3.6 Fluoroqinolone resistance mechanisms

Fluoroquinolones (FQs) inhibit DNA gyrase (topoisomerase II) and topoisomerase IV, resulting in microbial death [212]. *M.tb* does not have homologs of topoisomerase IV. DNA gyrase is an A_2B_2 protein. The A subunit carries the breakage-reunion active site and the B subunit promotes adenosine triphosphate hydrolysis. A and B subunits are encoded by *gyrA* and *gyrB*, respectively [213]. The quinolone-resistance-determining regions (QRDR) of *gyrA* (320bp) and *gyrB* (375pb) are the most important locations in FQ resistance. *GyrB* mutations are of much rarer occurrence and confer lower-level resistance than *gyrA* mutations [212]. The WHO definition of FQ resistance in *M.tb* is MIC of ofloxacin $\geq$ 2 µg/ml [214]. Other underlying mechanisms for mycobacterial resistance to FQs have been suggested such as decreased cell wall permeability to drug, drug efflux pumps, drug sequestration and drug inactivation [212, 215, 216]. A new proposed mechanism of quinolone resistance is related to *MfpA* which is a member of the pentapeptide repeat family of proteins from *M.tb*, whose expression causes resistance to FQs [217].

1.3.3.7 Resistance mechanisms of other second line agents

Ethionamide/prothionamide and thioamides: ETH is a derivative of isonicotinic acid and bactericidal with MICs of 0.5-2 µg/ml in liquid medium, 2.5-10 µg/ml in 7H11 agar and 5-20 µg/ml in LJ medium. ETH is a prodrug that is activated by *EtaA/EthA* (monooxygenase) and inhibits the same targets as INH through the mycolic acid synthesis pathway coded by *inhA*. Mutations in the drug-activating enzyme

EtaA/EthA cause resistance to ETH and other thionamides [218]. Mutations in *inhA* confer resistance to both INH and ETH. Due to the relative rarity of resistant strains and the lack of standardization and reproducibility of DST for the second-line agents, the resistance mechanisms are poorly characterized.

It is of vital importance that the mechanisms of resistance in *M.tb* to all drugs are fully elucidated to facilitate rapid diagnosis, drug class preservation, and the development of novel compounds. This is particularly important for the first line agents. Whole genome sequencing efforts are revealing further mechanisms of resistance but environmentally induced mechanisms will require *in vitro* characterization [189-191, 219].

1.3.4 Treatment of drug resistant tuberculosis

1.3.4.1 Treatment of MDR TB

MDR TB is a treatable disease under ideal conditions. In practice, only one in ten patients have access to treatment and the cure rates vary widely from 25% to 82% [79]. Adherence to treatment is an essential element for success. MDR patient cohorts can vary widely in resistance patterns with some regions having rare second-line resistance or exposure to second line drugs and other regions, such as the former Soviet states, having wide-scale resistance to multiple second-line drugs among MDR cases [79]. Treatment success is highly dependent on the extent of resistance to additional drugs. The absence of second- line drugs in previous regimens is strongly associated with a successful outcome of MDR-TB treatment [220]. Withdrawal of drugs due to adverse effects has been found to be related with poor treatment outcomes, due to the potential for further amplification of drug resistance. HIV

infection is also a strong risk factor for relapse. Among MDR TB patients in a cohort study in the US the overall relapse rate was 2.06 per 100 persons-years for HIV-infected patients compared with 0.52 for non-HIV-infected patients [221]. According to a systematic review and meta-analysis, overall the successful outcome rate for MDR treatment cohorts which have been reported is 62% [95% CI: 57-67] while 13% defaulted, 11% died, and 2% were transferred out [220]. Factors related to poor outcomes were male gender, alcohol abuse, low BMI, smear positivity at diagnosis, fluoroquinolone resistance and the presence of an XDR resistant pattern. Surgical intervention, no previous treatment and fluoroquinolone usage were factors associated with good outcome. Three categories of treatment regimen for MDR TB include standardized regimen, empirical regimen and individualized regimen. In standardized regimens, DST data from representative patient populations are used to design regimens in the absence of individual DST. Empirical treatment regimens are designed based on the TB treatment history of the individual patient and DST data from the representative patient population. Individualized treatment regimens are guided by the treatment history of the patient and individual DST results. It is important to consider the treatment history of a patient even when full DST results are available because DST can be unreliable, particularly for second-line agents, and may take many months to obtain, during which time the DST profile of the *in vivo* strain may have evolved.

Anti-TB drugs are divided into 2 principal categories: first- and second-line drugs (table 1.5). Researchers also classify agents into 5 groups based on efficacy, experience of drug usage and drug class.

Group 1: first-line oral agents including Isoniazid (INH), Rifampicin (RiF), Ethambutol (EMB), Pyrazinamide (PZA) and Rifabutin (Rfb).

Group 2: injectable agents such as kanamycin (KM), amikacin (AMK), Capreomycin (CPM) and streptomycin (SM).

Group 3: Fluoroquinolones (FQs) .

Group 4: oral bacteriostatic second-line agents such as ethionamide (ETH), prothionamide (Pto), cycloserine (Cs), terizidone (Trd) and p-aminosalicylic acid (PAS).

Group 5: Agents with unproven efficacy and/or high toxicity such as clofazimine (Cfz), linezolid (Lzd), amoxicillin/clavulanate (Amx/Clv), thioacetazone (Thz), Imipenem/cilastin (Ipm/Cln), high-dose Isoniazid and clarithromycin (Clr).

The basic principles for designing a MDR treatment regimen are : (1) the regimen should be based on the history of drugs that patients are known to have taken; (2) based on the prevalence of resistance to individual drugs in the population from which the patient originates; (3) At least four drugs should be included to which the isolate is thought to be susceptible; (4) PZA, EMB and a quinolone should be given once a day, in contrast, ETH, Cs, PAS are given in split doses; (5) the drug dosage should be determined by body weight; (6) treatment of adverse drug effects should be immediate and adequate; (7) an injectable drug should be used for at least six months; (8) the minimum length of treatment should be 18 months after culture conversion; (9) drugs are administered as directly observed therapy; (10) DST does not predict with 100% certainty the effectiveness or ineffectiveness of a drug; (11) PZA can be

used for the entire treatment and (12) early DR-TB detection and treatment are important factors.

Duration of treatment for MDR-TB is currently a minimum of 18 months after negative culture conversion. Extension of therapy to 24 months may be indicated in chronic cases with extensive pulmonary damage.

In Bangalesh, a 9-12 month regimen (4 months gatifloxacin, clofazimine, ethambutol, pyrazinamide, prothionamide, kanamycin and high–dose isoniazid/ 5 months gatifloxacin, clofazimine, ethambutol and pyrazinamide) gave a relapse-free cure of 87.9% (95% CI, 82.7-91.6) among 206 patients in a cohort analysis [222]. A RCT entitled Standardized Treatment Regimen of Anti-TB drugs for patients with Multi drug-resistant TB (STREAM) has been developed to test this regimen used in the Bangladesh study. The trial has a non-inferiority design to compare the Bangladesh regimen with the standard 18-month minimum WHO treatment regimen (Clinical Trial number: ISRCTN78372190). Vietnam is one of the countries participating in this trial. The clinical trials uses moxifloxacin in place of gatifloxacin due to the lack of a GMP source for gatifloxacin.

Due to the desperate need for improved, less toxic and shorter treatment regimens for MDR TB several countries have expressed an intention to implement the 'Bangladesh regimen' and WHO has therefore adopted a policy of offering technical advice on implementation and evaluation [223].

1.3.4.2 Novel TB drugs for MDR TB

In 2013 the diarylquinolone R207910 (bedaquiline also known as TMC 207 or J) was approved by the FDA and WHO as the first new drug class for the treatment of MDR TB [22]. Bedaquiline was shown in a phase 2 randomised controlled trial to reduce the time to sputum culture conversion (hazard ratio, 11.8; 95% CI: 2.3 to 61.3; p=0.003), and increase the proportion of patients with sputum culture conversion (48% vs. 9%), with no increase in mild and moderate adverse events except nausea occurred significantly more frequently among patients with Bedaquiline (26% vs. 4%, p=0.04) compared to placebo group [224]. Bedaquiline has also been tested in combination therapy which showed that it was well tolerated with safe treatment outcomes [225]. Five conditions necessary for WHO-supported use of bedaquiline are specified: effective treatment and monitoring, proper patient inclusion, informed consent, adherence to WHO recommendations and active pharmacovigilance and management of adverse events. The WHO guideline for interim use of bedaquiline emphasizes the need for further phase III evaluation of this drug [226].

Several new drugs for tuberculosis have been discovered and used off-label for MDR treatment moxifloxacin (M) and linezolid (Lzd). Rifapentine in the rifamycin class is also FDA approved for TB treatment. Further novel agents are at the preclinical and clinical trial stage (Figure 1.10), including the nitroimidazoles (PA 824 and delanamid/OPC 67683) (www.newtbdrugs.org).

Figure 1-10: TB drug pipeline 2013

Reproduced from Zumla et al. Nature Reviews Drug Discovery 2013 [227].

1.3.4.3 Regimens for drug-resistant TB

Regimens for the treatment of DR-TB are based on expert opinion, rather than evidence from randomized controlled trials. Recent meta-analyses of data from RCTs

have provided some evidence to guide treatment of DR-TB. INH-monoresistant TB has a good outcome if identified before the emergence of further resistance Short-course chemotherapy can achieve around 98% cure with less than 5% relapse when 4 drugs (RHZE/S) are used throughout the 6 months of treatment [228]. If the regimen is reduced to two drugs in the continuation phase after two months (RH), the relapse rate after 6 months rises to 10% [229]. Some researchers have advised alternative regimens such as RE or REZ for more prolonged durations. The best treatment for INH mono-resistant TB programmatically is still unclear.

RiF-monoresistant TB has a much more ominous prognosis. Some researchers have previously recommended a regimen with HZE for 18-24 months [230] but the addition of a fluoroquinolone to the regimen to complement INH in place of RiF is likely to be a more effective regimen [231]. RiF monoresistance is rare, but is more common among HIV-infected individuals, which complicates treatment. Research is required to determine the optimum regimen in these cases but, due to the rarity of the condition, it is likely that treatment regimens will continue to be guided by caution to prevent the development of MDR TB for the foreseeable future.

1.3.4.4 Surgery for MDR TB

Surgery may be considered in intractable cases of MDR TB to improve the efficacy of drug therapy but it is not curative and the threshold for surgical intervention varies widely between centres [232]. At least two months of drug therapy should be given before surgery to decrease the bacterial load with an additional 12-24 months of chemotherapy following surgery. Surgery is more effective in cases where the disease is limited to a single lung or lung lobe.

1.3.4.5 Treatment of DR-TB in special conditions and situations

Co-morbidities including diabetes mellitus, liver disorders, renal insufficiency, seizure and psychiatric disorders, complicate the treatment of drug-resistant TB and must be given consideration in the choice of drugs and monitoring of adverse events [65].

Pregnancy is not a contra-indication to treatment but injectable agents are not recommended. Lactating mothers should be given infant formula and attempts be made to limit exposure of any children in smear positive cases [65].

1.3.4.6 Monitoring progress of treatment of drug resistant TB: adverse effects

Second-line drugs have a higher range and frequency of adverse effects than the first-line antituberculosis drugs [65]. If adverse effects are mild and not dangerous, the treatment regimen should be continued with the help of ancillary drugs. Reducing dosages of the offending drugs is another method for managing adverse effects [233]. In principle, dosage should not be reduced below the level expected to produce adequate serum levels for TB treatment, however in practice this is difficult to determine, especially for the second line drugs. Sometimes, cycloserine and

ethionamide are completely tolerated at a lower dose. Pyridoxine should be given to any patient receiving cycloserine to prevent neurological adverse effects with a dose of 50 mg for every 250 mg of cycloserine prescribed. Psychosocial support is a component of the management of adverse effects [65, 234].

AUC = area under curve; MIC = minimum inhibitory concentration; EBA = early bactericidal activity; cfu = colony forming units; ? = uncertain; PAS = para-aminosalicylic acid.

Table 1-5: Pharmacokinetic characteristics of current anti-tuberculosis drugs

Reproduced from Mitchison et al. IJTLD 2012 [235]

1.4 INH Resistant Pulmonary Tuberculosis

1.4.1 Chemical characteristics of Isoniazid

Isoniazid was first synthesized in 1912 and first used clinically in 1952. The molecular formula is: $C_6H_7N_3O$ with a molecular weight of 137,14.

Generic names for isoniazid are isonicotinic acid hydrazide; isonicotinylhydrazine; isonicotinylhydrazine.

1.4.2 Mechanism of action

INH is a bactericidal agent against organisms of the genus Mycobacterium including *M.tuberculosis.* It has the highest early bactericidal activity (EBA) of the TB drugs and is responsible for the rapid decrease in bacterial load in the first week of treatment [236-239]. INH is bactericidal to rapidly-dividing *M.tb* bacilli but bacteristatic to replicating bacilli [240].

INH is a prodrug activated by the catalase-peroxidase haemoprotein product of *katG* and is transformed into iso-nicotinic acid, which has a similar structure to nicotinic acid, an important precusor of nicotinamide adenine dinucleotide (NAD). The accumulation of iso-nicotinic acid leads to the generation of iso-NAD instead of NAD which then blocks multiple enzyme systems as a non-functional co-factor [180]. Isoniazid acts in multiple pathways, principally to inhibit mycolic acid biosynthesis. Isoniazid also induces an activation of the mycobacterial NADase and leads to a diminution of the intracellular NAD concentration.

INH also inhibits inhA, a nicotinamide adenine dinucleotide (NADH)-specific enoyl-acyl carrier protein (ACP) involved in fatty acid synthesis.

1.4.3 Pharmacokinetics and Pharmacodynamics of isoniazid

1.4.3.1 Absorption

Isoniazid is a highly reactive water soluble drug absorbed from a limited area of the small intestine. INH should not be given with food because this significantly reduces its bioavailability. A high carbohydrate diet, high gastric pH and altered hepatic and gastric blood-flow may also decrease bioavailability of isoniazid [241].

Peak plasma concentrations are reached after 1-2 hours after ingestion and the median peak plasma concentration approximately 3μg/ml. It is lower in children than adults due to faster elimination, with the same mg/kg dosage [242-244]. The WHO treatment guidelines for pediatric TB have recently been revised to reflect this [245]. .

INH is distributed in all body fluids including cerebrospinal, pleural and ascitic fluids; and in tissues, organs and excreta.

1.4.3.2 Metabolism

INH is acetylated to N-acetylisoniazid (NAT), then biotransformed to isonicotinic acid and monoacetyl-hydrazine. An individual may be fast (FF), intermediate (FS) or slow acetylator (SS), depending on polymorphisms in the N-acetyl-transferase-2 genotype (NAT2) . Slow acetylators are characterized by a relative lack of hepatic N-acetyltransferase. A pharmacokinetic study showed that 88% of the variability in INH serum clearance is affected by NAT2 gene *4 (NAT2*4) alleles. Ethnicity, gender and body weight contribute relatively little to the variability [246]. In addition to

affecting the pharmokinetics of serum INH concentrations, differences in INH metabolism also generate different concentrations of toxic metabolites and may impact the rate of adverse reactions. Some researchers suggest that the rapid acetylators should take larger doses of INH than slow acetylators, who are at higher risk of adverse reactions such as peripheral neuritis, and hepatic toxicity [247].

The distribution of acetylator types varies across different ethnicities: in most Asian ethnicities the majority are fast acetylators, while among Caucasians the ratio is reversed [248]. NAT2 genotyping could become a useful alternative to therapeutic drug monitoring for INH [249].

According to a study conducted in Warsaw, with a 300mg dose of INH 38.6% of fast acetylators did not achieve a concentration of 1 μg/ ml in plasma three hours after ingestion of the drug. In contrast, 29.7% of slow acetylators maintained concentrations of INH 2 μg/ml in plasma six hours after ingestion [250] .

1.4.3.3 Excretion of INH

INH is primarily excreted through the kidney. Other routes are breast milk, saliva, sputum and faeces. About 50% to 70% of a 5mg/kg oral INH dose is excreted in urine within 24 hours as metabolites, varying by acetylator type [251].

1.4.3.4 Drug-drug interactions of INH

Known drug interactions for INH are attributable to its interaction with the cytochrome P450 system, especially CYP2E1. INH shows a biphasic inhibition-induction so it increases serum concentrations of some drugs such as phenytoin and carbamazepine. INH increases the effects of warfarin and theophylline, and inhibits

metabolism of benzodiazepines, monoamine oxidase and histaminases [252]. There are no known interactions with antiretroviral agents for HIV [67].

1.4.4 Toxicity of INH

1.4.4.1 Hepatitis

INH usage is associated with a risk of developing severe, potentially fatal hepatitis. The risk of hepatotoxicity increases with age, it is rare in persons under 20 but up to 2.3% in those who are over 50 years of age and daily alcohol consumption [253]. Acetyl hydrazine, which is released from acetylated INH, may be one of the toxic metabolites responsible for hepatitis with higher concentrations reached in slow acetylators. The symptoms of hepatitis are anorexia, nausea, vomiting, fatigue, malaise and weakness. Between 10-20% patients using INH have mild and transient elevation of serum transaminase levels which is not a contraindication to continued use [254].

1.4.4.2 Central Nervous System

Potential neurotoxic adverse reactions are convulsions, toxic encephalopathy, optic neuritis, optic atrophy, memory impairment and toxic psychosis. These side effects are not common in TB treatment but may occur in cases of overdose [255].

Use of INH can produce peripheral neuropathy but this can be prevented by concurrent administration of pyridoxine. Peripheral neuropathy is a dose-dependent adverse reaction that occurs in less than 0.2% of patients at dosages of 3-5 mg/kg/day and more commonly in patients with malnutrition, diabetes, HIV infection, renal failure, alcoholism, pregnancy or lactation. Pyridoxine should be given at a dosage of

5-10 mg/ day for prevention and at 250mg/day for treatment of isoniazid-induced neuropathy [256, 257].

1.4.4.3 Other adverse events associated with INH

Potential gastrointestinal adverse effects include nausea, vomiting and epigastric distress. Rare haematological adverse effects include agranulocytosis, haemolysis, aplastic anaemia, thrombocytopaenia and eosinophilia.

Endocrine and metabolic effects which can occur but are also rare are pellagra hyperglycaemia, acidosis and gynecomastia.

Hypersensitivity reactions occur in relatively rare cases (<1% of patients) and manifest as fever, skin rashes, lymphadenopathy and vasculitis [258].

Development of antinuclear antibodies occurs in 1 of 5 patients receiving isoniazid. INH should not be used in patients who develop clinical lupus erythematosus (less than 1%).

1.4.5 The early bactericidal activity (EBA) of INH

EBA is defined as the fall in counts/ml sputum/day during the first 2 days of treatment. INH at a 300 mg dose has the highest EBA of any antituberculous agent. The bactericidal activity of INH is dose- related. The N-acetyl-transferase-2 genotype (NAT2) also influences the EBA of INH; faster INH acetylators have been found to have a lower EBA [131].

1.4.6 Treatment of INH resistant pulmonary TB

Resistance to INH is the most common form of drug resistance and the prevalence of INH resistance (alone or in combination with other drugs) in newly pulmonary TB

was estimated at 10.3% in the world in 2008 [259]. INH resistance is defined as low level resistance and high level resistance if there is over 1% growth of *M.tb* in the presence of 0.2 μg/ml and 1 μg/ml, respectively. INH resistance is a key precursor in the development of MDR-TB. The efficacy and optimal duration of treatment for INH resistant new pulmonary TB patients have long been discussed. Treatment failure, relapse and acquired resistance to other first-line drugs such as RiF may occur. There is no evidence from definitive randomized controlled trial to determine the best treatment for various patterns of drug resistance [260] .

Several studies have demonstrated that there is an association between the *katG* S315T mutation and high-level INH resistance, and an association between *inhA* mutation and low-level INH resistance [3, 172, 177, 178]. A US study concluded that there was no association between INH resistance mutation and clinical presentation but this is not surprising and the impact on treatment outcomes has not been established [261]. A study in Korea evaluated alternative treatment regimens for INH-resistant pulmonary TB such as 2RHZE/10RE, 2RHZE/7RE and 2RHZE/4REZ. They concluded that the overall success rates were 90% for 2RHEZ/10RE, 92% for 2RHEZ/4REZ and 100% for 2RHEZ/7RE. However, this study was too small to draw robust conclusions (n=39 patients) [262] and efficacy under study conditions is likely to vary substantially from effectiveness under programmatic conditions. From a study conducted by Ormerol *et al*, a regimen of 2RZE/7RE may be possible for the treatment of isoniazid-resistant organism if given under close supervision [263]. In another study in Southeast Texas, several INH–resistant treatment regimens were also compared. They concluded that treatment durations should be over 7 months, and

twice-weekly treatment regimens were associated with relapse, whereas thrice-weekly and daily treatments performed similarly [264]. According to WHO, regimens that are suggested for INH resistant pulmonary TB (+/- SM resistance) consist of RiF, PZA and EMB for a minimum duration of treatment of 6-9 months. They advise that a fluoroquinolone may be added to the regimen to strengthen it for patients with extensive disease [260] . Thus, there is no clear evidence of the optimal regimen for INH-resistant TB and adequately powered randomized controlled trials are needed.

1.5 Anti-TB drug-induced hepatotoxicity

The most frequent adverse reactions to TB treatment are hepatotoxicity, skin reactions, and gastrointestinal and neurological disorders [265]. Anti-TB drug-induced liver injury (DILI) or anti-TB drug-induced hepatotoxicity (ATDIH) is a significant problem which causes substantial morbidity, and mortality of 6%-12% if TB drugs are continued after the onset of symptoms [233]. Asymptomatic transaminase increases are common during TB treatment but hepatotoxicity can cause death if it is not recognized early. Adverse effects in general, and ATDIH in particular, will diminish treatment effectiveness because of nonadherence, and they may eventually contribute to treatment failure, relapse or drug-resistance if poorly managed. Isoniazid, rifampicin and pyrazinamide, which are metabolized by the liver, are potentially hepatotoxic drugs.

1.5.1 Functions of the liver

The liver has a central role in drug metabolism and detoxification. After drugs are ingested, the splanchnic circulation carries them directly into the liver. Metabolic

enzymes convert them through three pathways. Most anti-TB drugs require biotransformation with phase 1 and phase 2 enzymes in order to become water-soluble compounds. The phase 1 pathway makes use of enzymes for oxidation, reduction and hydrolysis of the cytochrome P450 class. The Phase 2 pathway includes glucuronidation, sulfation, deacetylation and deamination which forms compounds ready for excretion. In the phase 3 pathway, the cellular transporter proteins excrete these compounds into bile or the systemic circulation. Activity of these pathways is influenced by both endogenous and exogenous factors, including circadian rhythms, hormones, cytokines, morbidities, genetic factors, general health and nutrition status of an individual and presence and dose of chemicals.

In phase 1, oxidation or demethylation that occurs with the aid of cytochrome P450 (CYP450) enzymes which usually produces toxic intermediates. In phase 2, a large water-soluble compound that transformed by glucuronidation or sulfation results in eliminated non-toxic metabolites. Glutathione, an enzyme in second phase, is responsible for detoxification, for example, enzyme glutathione S-transferase can bind to toxic compounds. Transporters, such as P-glycoprotein, nuclear receptors and pregnane X-receptor, also play a critical role in the orderly elimination of drugs during phase 3 metabolism.

Figure 1-11: Six mechanisms of liver injury

Reproduced from Lee, NEJM 2003 [266].

1.5.2 Definition of Antituberculous drug-induced hepatotoxicity

ATDIH is a clinical diagnosis of exclusion because histological samples of the liver are usually not obtained. Acute viral hepatitis should be considered as a differential diagnosis. The signs and symptoms of ATDIH are jaundice, abdominal pain, nausea, vomiting, asthenia, encephalopathy and fulminant hepatic failure. ATDIH ranges from nonspecific transaminase increase to fuminant hepatic failure. ATDIH is potentially fatal if not detected and managed appropriately [267]. The time of onset to acute liver injury is usually within one month of initiating antituberculous drugs but may occur later. Many definitions of ATDIH have been used in the literature but a consensus definition has not been reached leading to subjectivity in diagnosis and treatment. A common definition is an increase in serum alanine aminotransaminase (ALT) greater than three times the upper limit of normal (ULN) with symptoms of hepatitis, or five times without symptoms; and/or an increase in serum total bilirubin over 1.5mg/dl (1 mg/dl x 17.1= μmol/l) and this is the definition used in this thesis. A recent consensus statement on management of HIV/TB also includes total serum bilirubin over 40 μmol/l as a criteria for ATDIH [268]. For patients with increase of liver function tests (ALT) before TB treatment, the elevation of ALT greater than 1.5 times the baseline ALT should be considered as ATDIH. An increase in serum ALT (SGPT: serum glutamate pyruvate transaminase) is more specific than an increase in AST (SGOT: serum glutamic oxaloacetic transaminase) for hepatocellular injury, because abnormalities in muscle, heart or kidney also show an increase in AST [269]. ALT and AST levels rise following exercise, haemolysis or muscle injury and tend to

be higher in men, especially in persons with high body mass index. Children and the elderly tend to have lower transaminase concentrations. AST is higher than ALT in alcohol-related transaminase elevation.

Increase in alkaline phosphate and/or bilirubin, with little or no increase in ALT, suggests cholestasis. An increase in serum γ-glutamyl transpeptidase can differentiate liver-related AP increase from other organ-related AP increases. Jaundice is usually detected when serum bilirubin exceeds 3.0 mg/dl (normal total bilirubin is from 0.3 to 1.9 mg/dl) (μmol/L x 0.0585 = mg/dl).

Rechallenge with the suspected agents with ALT elevation more than two times of ULN and a fall in ALT with discontinuation of suspected agents may strongly support the diagnosis. Time interval from initiation of anti-TB drugs to occurrence of ATDIH is considered as the latency period, which usually ranges from 6 to 102 days (mean: 17.35 +/- 18.67 days). The time interval between stopping INH, RiF and PZA and achieving normal liver function tests is considered as the normalization period, which usually ranges from 4-58 days (mean: 18.71 +/- 11.36 days) [270].

1.5.3 Incidence

The incidence of ATDIH during TB treatment has been reported at between 2% and 28%. The incidence varies between different world regions. Asian countries, especially underdeveloped regions where factors such as liver disease, malnutrition and advanced TB disease play a role, are reported to have the highest rates of incidence of ATDIH [271]. From four Indian studies, the risk of ATDIH was 11.5% in India compared to 4.3% in Western countries [272]. Transaminase elevations are reported in 0.3% of all patients treated with isoniazid monotherapy [273]. In a trial of

a novel rifampicin and pyrazinamide prophylactic regimen for latent TB, hepatotoxicity occurred in 5.3% of patients [274]. When pyrazinamide was introduced in the 1950s, a high incidence of ATDIH was reported. This appears to be associated with the high dosage of PZA used in earlier studies of 40-70 mg/kg [275], however, PZA is responsible for more hepatotoxicity than INH and RIF in current regimens.

1.5.4 Pathological features

INH-induced hepatotoxicity manifests as hepatocellular steatosis and necrosis, and toxic isoniazid metabolites bind to cell macromolecules. Hydrazine, which is the toxic metabolite of INH, causes steatosis, hepatocyte vacuolation and glutathione depletion. Lipid vacuoles and mitochrondrial swelling are found in periportal and midzonal hepatocytes.

Hyperbilirubinemia is a common manifestation due to interference with bilirubin excretion. Centrilobular necrosis is a lesion caused by RiF, possibly associated with cholestasis. Lesions range from spotty to diffuse necrosis to cholestasis [276].

In patients who have died of RiF- and PZA-induced hepatotoxicity, lesions are found, such as bridging necrosis, lymphocytic infiltration, focal cholestasis, increased fibrosis and micronodular cirrhosis [66].

1.5.5 Mechanism of toxicity

ATDIH may result from three main mechanisms: (1) from a metabolite to cause direct toxicity, and/or (2) from an immunological response affecting hepatocytes, biliary epithelial cells, and/or (3) from idiosyncratic reactions, which are independent

of dosage, to affect liver vasculature [233]. Idiosyncratic reactions comprising most types of ATDIH may result in hepatocellular injury and/or cholestasis. In hypersensitivity reactions, haptens are produced by immunogenic drugs or their metabolites are free or bound to hepatic proteins. The processes including antibody-dependent cytotoxic, T-cell and eosinophilic hypersensitivity responses, may be evoked. Tumor necrosis factor-α, interleukin (IL)-12 and IFN- γ are released and promote hepatocellular programmed cell death (apoptosis), an effect opposed by IL-14, IL-10, IL-13 and monocyte chemotatic protein-I. Idiosyncratic reactions may recur within days to weeks after re-exposure.

The exact mechanism of ATDIH is unknown if it is due to idiosyncratic reaction, it can affect any organ system. Figure 1.11 shows six possible mechanisms of liver injury.

1.5.5.1 Isoniazid

Acetylation is the main metabolic pathway of INH metabolism. INH (isonicotinic acid hydrazide) is acetylated by the hepatic enzyme N-acetyltransferase 2 (NAT-2) into acetylisoniazid, which is then hydrolyzed into acetylhydrazine and isonicotinic acid. Acetylhydrazine is either hydrolyzed into hydrazine or acetylated into diacetylhydazine and is subsequently oxidized into hepatotoxic intermediates by cytochrome P450 2E1 (CYP2E1). A small part of INH is directly hydrolyzed into isonicotinic acid and hydrazine [277].

Previously, it was thought that acetylhydrazine was the toxic metabolite of INH but recently, researchers have suggested that hydrazine, not INH and acetylhydrazine, is more likely to be the cause of INH-induced hepatotoxicity [277]. Several hydrazine

metabolites, such as acetylhydrazine, hydrazones and nitrogen gas, have been identified. The major route of hydrazine metabolism is oxidation. In hydrazine reactions; nitrogen, diimide and powerful diazene reducing agents; are intermediates. One study on rat liver microsomes showed that nitrogen-centered radicals, which formed during oxidative hydrazine metabolism, probably are involved in the hepatotoxic process [278].

Cytochrome P450 2E1 (CYP 2E1) is involved in ATDIH because it is associated with the metabolism of several carcinogens and drugs, and it may be related with susceptibility to alcoholic liver disease and many types of cancers such as hepatocellular carcinoma. Three genotypes of CYP 2E1 are c1/c1, c1/c2 and c2/c2. The CYP 2E1 c1/c1 genotype is related to high CYP 2E1 activity and may produce many hepatotoxins. Animal studies showed that isoniazid and hydrazine induce CYP 2E1 activity and INH inhibited effects on CYP1A2, 2A6,2C19 and 3A4 activity. CYP1A2 is involved in hydrazine detoxification. CYP2E1 is related to the severity of ATDIH [279].

Oxidative stress, which results from an imbalance between oxidants and antioxidants involving ATDIH, is still a topic for debate. Non-enzymatic and enzymatic systems (e.g. glutathione conjugation) are associated with the detoxification of reactive oxygen species. Reduced glutathione levels, activity of glutathione-S transferase, catalase and superoxide dismutase after the administration of INH and hydrazine, show that oxidative stress is involved in INH-induced hepatitis. N-acetylcysteine (a sulfhydryl-containing compound) has a hepatoprotective effect. Futhermore, TB patients with ATDIH have lower plasma levels of glutathione and higher

malondialdehyde, which is an oxidative stress parameter. Induced glutathione depletion is not directly involved in INH-induced hepatitis. One study from China showed that the glutathione S-transferase mu 1 (GSTM1) Rsal null genotype tended to increase susceptibility to ATDIH but the association with ATDIH was not significant [280].

1.5.5.2 Rifampicin

RiF is a potent inducer of cytochrome P450. The major pathway of RiF metabolism is desacetylation into desacetylrifampicin and hydrolysis will produce a 3-formyl rifampicin. The mechanism of RiF-induced hepatitis is unknown. The combination of RIF and INH has been associated with an increased risk of hepatotoxicity because RiF induces isoniazid hydrolase which increases hydrazine production, especially in slow acetylators [281] (figure 1.12). ATDIH of RiF has been detected in patients with underlying liver diseases. RiF is implicated in cholestatic ATDIH [282].

Figure 1-12: Rifampicin induction of the hydrolysis pathway of isoniazid metabolism into the hepatotoxic metabolite hydrazine.

Reproduced from Askgaard et al. Thorax 1995 [281]

1.5.5.3 Pyrazinamide

PZA (pyrazoic acid amide) is converted into pyrazinoic acid (PA) and then is oxidized to 5-hydroxypyrazinoic acid by xanthine oxidase. A report of a cell- line, animal and clinical trial from Taiwan confirmed that 5-hydroxypyrazinoic acid (5-OH-PA) was responsible for PZA-induced hepatitis [283]. The mechanism of PZA-induced hepatotoxicity is unknown, but it is possibly due to direct toxicity. A study

from Taiwan showed that slow acetylators developed ATDIH more frequently in anti-TB treatment with first-line (INH+RiF+PZA+EMB) drugs, specially in a regimen in which PZA is administered at the same time as INH, RiF and EMB [279]. The mechanism of hepatotoxicity of PZA is both dose-dependent (5% for a dosage of 20-30 mg/kg per day and 10% for a dosage of 40-70 mg/kg per day) [284] and idiosyncratic. In a cohort and case-control analysis, researchers found that adding PZA to INH and RiF would increase the risk of ATDIH appreciably [285].

1.5.5.4 Other drugs

ATDIH occurs in about 2% of patients treated with ethionamide or prothionamide, and in 0.3% with PAS. Cycloserine does not appear to be associated with ATDIH [65].

1.5.6 Risk factors for anti-tuberculous drug induced hepatotoxicity

Many risk factors for ATDIH have been reported. Older patients are at higher risk of hepatotoxic reactions due to the decreased clearance of drugs metabolized by CYP450 enzymes and changes associated with ageing in liver blood flow, liver size, drug binding. Heptotoxicity of antituberculous drugs is 4 times higher in patients over 35 years of age [286].

The risk of hepatotoxicity may be higher in women due to higher CYP 3A activity but studies have reported conflicting findings [287]. According to one study from Taiwan, abnormal baseline transaminase levels are an independent risk factor for ATDIH [288].

Extensive TB disease may be another risk factor for ATDIH. Minimal disease is defined as TB lesions that involve only one lobe or there is no cavitary lesion on chest

radiography. Extensive disease is defined as TB lesions that occupy more than two lobes, or in both lungs, or have cavitary or miliary images on chest X ray film.

Patients with HIV have altered activities in the oxidative pathway so they are more likely to have ATDIH. HIV patients require concomitant use of anti-TB drugs and anti-retroviral (ARV) drugs which also have hepatotoxic effects. The use of antifugal drugs (for example, fluconazole) is also a risk factor for ATDIH.

Several studies show that hepatitis B and C coinfection increase the risk of ATDIH. In general, patients with pre-existent liver disease (liver cirrhosis, fatty liver or diffuse hepatic pathology) are at risk of hepatotoxicity [289]. In Taiwan, high initial HBV/HCV viral load was a significant predictor of ATDIH [290]. From a study in Korea, researchers found that ATDIH occurred more frequently in HCV-seropositive patients during TB treatment [291]. However, using regimens containing RiF, INH, EMB and /or PZA after recovery from ATDIH, could be safe and successful [291]. Coinfection with hepatitis C and HIV elevates the risk of ATDIH. Acute viral hepatitis is a confounding factor in patients with ATDIH. ALT, AST, bilirubin levels are significantly higher in patients with acute viral hepatitis, compared with patients with ATDIH and time taken for normalization of liver functions was significantly longer in patients with acute viral hepatitis compared with patients with ATDIH.

The presence of HLA-DQB1*020 and the absence of HLA-DQA1*0102 were independent risk factors for ATDIH with OR of 1.9 and 4.0, respectively in North Indian patients [292].

In some studies but not in others, slow acetylator genotype is a risk factor for ATDIH. In one study from Turkey, researchers found that slow acetylators (76.7%) have

higher risk of developing hepatotoxicity than intermediate (13.3%) and fast acetylators (10%) with statistically significant difference ($p<0.001$). They concluded that analysis of NAT2 polymorphism could be used before treatment in hospital to determine which patients are at risk of developing ATDIH [293] .

The cytochrome P450 2E1 (homozygous wild type) and the glutathione S-transferase (homozygous null genotype) are related to ATDIH. Genetic polymorphisms in the pregnane X-receptor (PXR) have a role in CYP3A4 expression and in theory, they are involved in susceptibility to ATDIH.

Alcoholism is related to a higher risk of ATDIH because of enzyme induction. Daily TB treatment also increases the risk of ATDIH compared with thrice-weekly treatment but also increases the impact of intermittent non-adherence and the risk of treatment failure. Although some studies showed that alcohol use was a significant predictor of ATDIH, others found no association, although definitions of excessive alcohol consumption vary between studies [233].

Malnutrition or hypoalbuminemia (serum albumin levels of less than 3.5 mg/dl) is a risk factor for ATDIH. Patients with low albumin (<3.5 mg/dl) have been reported to have three times higher risk of ATDIH [294]. Body mass index (BMI= body weight (kg) / (height (m))^2) of 20kg/m^2 or less is an independent predictor of ATDIH, probably due to the depletion of glutathione stores and impaired drug metabolism by the liver [295].

1.5.7 Management

The management of ATDIH is shown in figure 1.13. In the cases of moderate or severe drug-induced hepatotoxicity, treatment should be interrupted and after hepatitis has resolved, treatment should be reintroduced. Guidelines differ on the pattern of reintroduction and in practice many clinicians follow protocols derived from individual experience and the clinical history of the patient. British Thoracic Society (BTS) and American Thoracic Society (ATS) guidelines recommend reintroducing drugs sequentially while the Task Force of the European Respiratory Society recommends restarting all drugs simultaneously [296]. According to a study, researchers found that the recurrence rate of ATDIH was not significantly different between the 3 different reintroduction regimens [292] .

The majority of patients have successful reintrodution of anti-TB drugs and tolerate reintroduction of INH, RiF and PZA. Another study showed that the risk of developing ATDIH after reintroducing all anti-TB drugs (RiF,INH,PZA) was 24% [270]. However, reintroducing INH, RiF and PZA after normalization of liver function tests should be approached with caution in patients with more severe ATDIH. After reintroduction of TB drugs, regular monitoring of liver function tests such as serum ALT, AST, and bilirubin levels should be done every week for the first month, every 2 weeks in the second month, and at the end of the third month. After the third month, laboratory testing should be performed when indicated.

Regimen selection for patients with ATDIH: In regimens without PZA, RiF, INH and EMB might be used for 9 months until DST of the *M.tb* isolate is completed.

In patients with cirrhosis, RiF and EMB and quinolones (levofloxacin, ofloxacin and gatifloxacin) or cycloserine should be used for 12-18 months. For patients with encephalopathic liver disease, the regimen consists of EMB, fluoroquinolone, cycloserine and capreomycin (or aminoglycoside) for 18 and 24 months. In one study from the USA, the researchers investigated the regimen of ethambutol, ofloxacin and streptomycin (EOS) for the first three months and nine months in continuation phase in patients with liver injury (3SEO/9EO) and they concluded that in the patients with severe liver disease, this regimen was well-tolerated and effective in 85% of patients [297]. Potential side effects with fluoroquinolone are nausea and vomiting, severe anaemia, conjunctivitis, pruritic rash, convulsions, arthralgia, vision change and giddiness [298].

Figure 1-13: Monitoring for hepatotoxicity during treatment of TB disease

Reproduced from Saukkonen *et al.* AJRCC2006 [233]).

1.6 Hepatitis B, C and Pulmonary Tuberculosis

1.6.1 Hepatitis B virus infection and pulmonary tuberculosis

Hepatitis B virus (HBV) belongs to the family of hepadnaviruses with a relaxed circular, double stranded DNA genome of approximately 3,200 base pairs [299].

Hepatitis B virus (HBV) infection is a major health problem and one of the most important infectious diseases in the world. It is estimated that about 2 billion people are infected, with over 350 million chronic cases and 1-2 million deaths per year. In Asia, the prevalence of chronic HBV infection is over 8% [300].

HBV is transmitted by perinatal, percutaneous and sexual exposure, or by person to person contact through open cuts and sores, transfusion, nosocomial infection and organ transplantation. HBV infection is more common in certain groups such as injecting drug users, persons with multiple sex partners, men who have sex with men, and workers who are exposed occupationally to contaminated blood and blood products [301]. Carriers of HBV are at higher risk of developing cirrhosis, hepatic decompensation and hepatocellular carcinoma [302]. Surface antigen (HBsAg) is detectable in the serum between 30 and 60 days post-infection. Anti-HBV surface antigen antibodies may develop after resolving infection or following vaccination and therefore a positive antibody test does not define infection. Anti-HBc antibodies develop in all HBV infections and persist indefinitely.

In the acute phase following infection the incubation period lasts from one to four months with a serum sickness-like syndrome manifesting with fever, skin rash, arthralgia and arthritis. The symptoms of hepatitis are right upper quadrant

discomfort, nausea, jaundice and other unspecific constitutional symptoms. Alanine and aspartate aminotransferase concentration levels may rise to 1000-2000 IU/L. Fulminant hepatic failure occurs in approximately 0.1-0.5% of patients [303].

Hepatitis B prevalence is highest in sub-Saharan Africa and East Asia and the rate of HBV chronicity of the adult population of these regions is between around 5%-10% according to WHO data. Most patients with chronic hepatitis B are clinically asymptomatic, with normal physical examination but some have symptoms of fatigue. If symptoms are present they may include stigmata of chronic liver disease, splenomegaly, spider angiomata, caput medusae, palmar erythema, testicular atrophy, gynaecomastia, jaundice, ascites, peripheral oedema and encephalopathy with decompensated cirrhosis. AST and ALT enzymes show mild to moderate elevation. The HBV virus may be in either a high replicative state or low/non-replicative state during chronic infection [301]. A high replicative state results in active liver disease, in which elevated serum ALT, HBV DNA and HBeAg are present. For the low or non-replicative phase, ALT may be normal, HBeAg is not detectable but anti-HBeAg antibody is present.

Inactive HBsAg carrier state is defined as (1) positive for HBsAg, (2) negative for hepatitis B e antigen (HBeAg) and positive for the antibody to HBeAg, (3) $<10^5$ copies per ml serum HBV DNA, and (4) normal pretreatment aspartate aminotransferase (AST)/ alanine aminotransferase (ALT) levels. Chronic hepatitis B is diagnosed according to the following criteria : (1) positive for HBsAg, (2) $>10^5$ copies per ml of serum HBV DNA and/or elevated AST/ALT levels before

undergoing antituberculosis treatment. Acute hepatitis B is diagnosed by testing for antibody to hepatitis B core antigen IgM (anti-HBc IgM) positivity [304].

Pulmonary tuberculosis patients with HBV are more likely to be sensitive to hepatotoxic anti TB drugs such as RiF, INH and PZA because of pre-existing hepatic damage, and their liver functions improve more slowly [303].

Patients with HBV infection have consistently been shown to have a higher rate hepatotoxicity on TB treatment, although the differences do not always reach statistical significance, probably due to study size. According to a study, in Brazil HBV infection showed considerably high relative risk (RR=2.91) although not statistically significant for anti -TB drugs induced hepatotoxicity. Using a scoring system and drawing a monograph, the study also shows how to estimate survival probabilities and median times to hepatotoxicity event for each patient [305]. In another study conducted in China in 2005, it was shown that the rate of hepatotoxicity in TB patients with HBV infection during TB treatment was higher than that in patients without HBV (59% vs 24%) [306]. Another study from Korea also showed that drug- induced hepatotoxicity occurred more frequently in HBsAg carriers than in control patients (8% vs 4%), although this was not a statistically significant discrepancy. However, the study showed HBsAg carriers with TB could follow standard regimens using RiF, INH, PZA and EMB by monthly liver function testing [307]. HBV coinfection in patients with HIV and TB is a risk factor for the development of severe hepatotoxicity, in which case highly active antiretroviral therapy (HAART) [308] and anti-TB drugs need to be stopped . In patients who are hepatitis B carriers receiving TB drugs, most liver dysfunction episodes are often

preceded by an increase in HBV-DNA levels and as a result, it may be better to consider the use of antiviral drugs such as lamuvidine to bring these levels down [309]. With these patients, regular liver function and drug monitoring are essential to detect asymptomatic cases before they evolve because early drug modification or withdrawal may prevent further liver damage and mortality.

1.6.2 Hepatitis C virus (HCV) infection and pulmonary tuberculosis

HCV, a member of the Flaviviridae family, is a small-enveloped virus with one single-stranded positive-sense RNA molecule of 9.6kb [310]. HCV is a major cause of progressive liver disease [311]. There are an estimated 170 million cases worldwide of which 80% are chronic. The complications of HCV infection are severe liver fibrosis and cirrhosis. 30%-50% of individuals with cirrhosis will develop hepatocellular carcinoma [312, 313]. Transmission usually occurs via sexual intercourse with HCV-infected individuals or shared needles. A systematic review showed that less than 1% of subjects who are exposed to HCV through contaminated needle stick injury develop acute HCV infection [312]. The level of HCV viremia can influence the risk of infection. Specific HLA types of children can influence the risk of acquiring infection from infected mothers [312].

Common symptoms of hepatitis C are fatigue, muscle ache, loss of appetite or nausea. It is estimated that only one out of four individuals infected with HCV is aware of the disease, so HCV diagnostics should be performed in all patients with increased aminotransferase levels [314]. HCV RNA measurement can discriminate between chronic hepatitis C and resolved HCV infection. Acute hepatitis C is a condition in

which both anti HCV antibodies and HCV RNA are positive but this may also occur in cases of acutely exacerbated chronic hepatitis C.

A study from Taiwan showed that HCV co-infection was an independent risk factor for hepatitis during anti-tuberculosis treatment and heaptotoxicity exacerbation was associated with slower recovery from hepatitis [315]. According to Ungo *et al.*, 30% of HCV-infected patients developed hepatotoxicity compared to 11% of non-HCV-infected patients during antituberculosis treatment. This study also showed that transaminases in HCV may be normal, or wax and wane over time in a "sine wave" pattern [316]. Kwon *et al.* also found liver enzyme levels were more often elevated during TB treatment in HCV-positive patients (41%) than in control subjects (20%), and hepatotoxicity occurred more frequently in HCV-infected patients (13%) compared to controls (4%). However, they also concluded that the reintroduction of TB therapy with RiF, INH, PZA and EMB could be safe and successful even in patients with HCV infection, but monthly liver function tests should be performed [291].

AIMS OF THE THESIS

1) Determine the rate of unfavourable outcome for patients with INH^R TB on standardised treatment outcomes.
2) Investigate bacterial factors associated with treatment failure for patients with INH^R TB.
3) Determine the prevalence of hepatitis B/C infection and the incidence of antituberculous drug induced hepatitis among TB patients in Ho Chi Minh City.
4) Investigate the impact of host factors, acetylator status and viral hepatitis coinfection on treatment outcome for patients with INH^R TB.

Chapter 2

Methods

2.1 Study design

2.1.1 Setting

Pham Ngoc Thach Hospital is responsible for TB control in HCM City and the southern provinces. It is a tertiary referral hospital and also functions as a primary TB hospital serving the local community, with around 750 staff and over 800 beds. In 2012, the hospital served 23,342 in-patients and 270,035 patients in total.

In Ho Chi Minh city, there are 24 District TB Units (DTU) namely districts 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, Binh Chanh, Binh Tan, Binh Thanh, Can Gio, Cu Chi, Go Vap, Hoc Mon, Nha Be, Phu Nhuan, Tan Binh, Tan Phu and Thu Duc (15 urban districts and 9 rural districts). DTUs take responsibility for the diagnosis and management of TB patients. The number of staff at each DTU is dependent on the patient numbers but may include doctors, nurses, technicians and social workers. Diagnosis of TB at DTUs is a passive process. When patients have symptoms suggestive of TB, they self-present at the DTU for sputum examination and chest X ray. DTUs also have facilities for acid fast bacilli (AFB) direct smear. If a DTU is located within a hospital, the other laboratory tests are available. However, culture and DST are not available in any DTU. Sputum samples are transferred to PNT Hospital for testing if needed. In case there are any difficult cases in diagnosis, treatment and management such as how to handle side effects, staff at DTUs will seek

advice from PNT Hospital doctors. Patients may self-present at PNT Hospital for diagnosis and initiation of treatment but are generally referred to their home DTUs for continued management once stable.

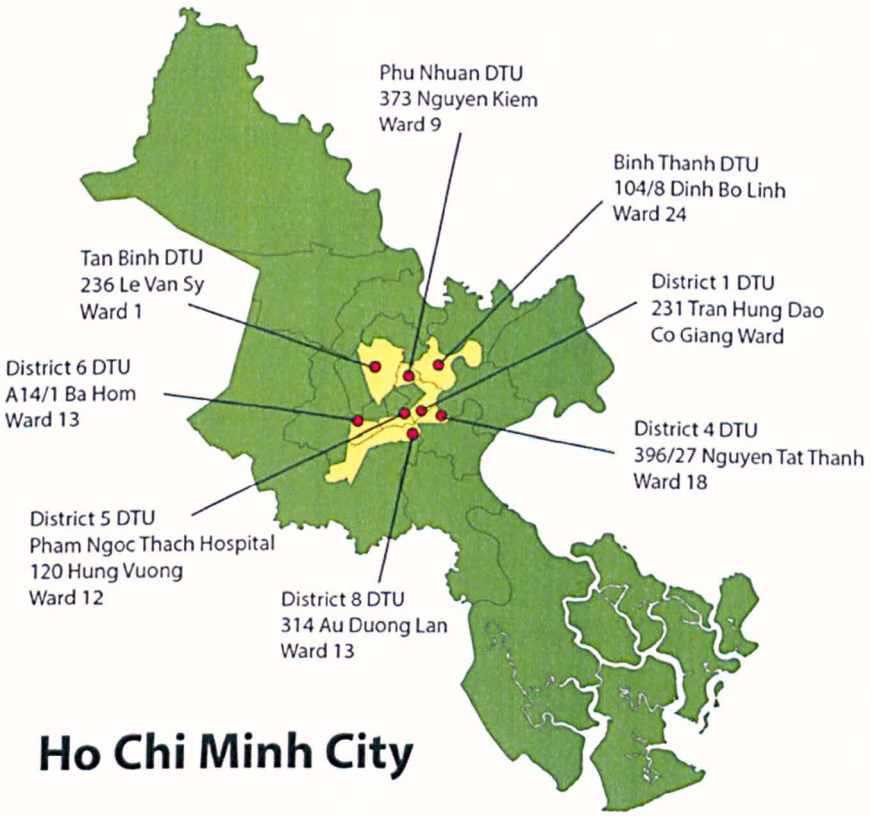


Figure 2-1 :Map showing geographic location of participating districts in Ho Chi Minh City

2.1.1.1 Participating sites

Four DTUs (district 6, 4, Binh Thanh and Phu Nhuan) and the out-patient department at PNT Hospital were initially chosen for participation in the study based on staff resources and patient burden (figure 2.1). Recruitment at these sites commenced in December 2008. After one year of recruitment, the number of INH resistant pulmonary TB patients fell short of the expected target. In October 2009, four additional DTUs (district 1, 3, 8 and Tan Binh) were invited to join the study and recruitment at these sites began in December 2009. The study completed recruitment at all sites in June 2011. District 3 was withdrawn from the study in early 2010 due to low recruitment (9 patients).

In summary, eight units participated in the INH study namely PNT Hospital out-patient department, district 6,4,1,8, Phu Nhuan, Binh Thanh and Tan Binh. Table 2-1 shows the number of health staff in DTUs participating in the study.

District	Doctor	Nurse	Nurse assistant	LAB technician	Others	Total	New smear positive pulmonary TBcases/ year
District 6	2	1		2	1 pharmacist of middle degree.	6	300
District 4	1			2	2 physician assistant 3 pharmacist of middle degree	8	363
Binh Thanh	1	3		2	2 pharmacist of middle degree. 2 physician assistant. 1 midwife	11	416
Phu Nhuan	1	2		1	2 physician assistant	6	140
Pham Ngoc Thach	13	36	6		3 administrative staff.	58	149
District 1	2	3		1	3 physician assistant. 1 nurse assistant	10	177
District 8	1	3		2	1 pharmacist 3 physician assistant	10	456
Tan Binh	1	3		2	1 physician assistant 1 pharmacist of middle degree 1 nurse assistant	9	252

Table 2-1: The number of health staff in DTUs participating in the study and the number of patients with new smear positive pulmonary TB in 2007

2.1.2 Sample size

It was estimated that approximately 25% of primary isoniazid-resistant cases would fail or relapse [317], half of which would be primary MDR-TB; 12.5% of primary isoniazid-resistant, non-MDR cases were therefore expected to fail treatment. Screening 2,200 patients was expected to yield approximately 550 isoniazid-resistant cases. With an anticipated 10% drop-out rate [37] [318], there would be follow-up data on 500 isoniazid-resistant cases. Approximately 200 (40%) of these patients would be infected with Beijing genotype strains. The study would therefore have 90% power to detect a 2-fold increase in failure rate among isoniazid-resistant Beijing genotype isolates.

2.1.3 Recruitment

From December 2008 to June 2011, all adult patients presenting to the units (districts 6, 4, Phu Nhuan, Binh Thanh and PNT Hospital out-patient department) who were newly positive smear pulmonary TB cases, were asked to participate in a screening study. From November 2009 to June 2011 we sped up the recruitment by including 4 further districts (district 1,8,3 and Tan Binh) in the study. All patients presenting to these units were evaluated for inclusion in the screening study and invited to participate if eligible. If the patients agreed, one sputum sample for MODS and a sample of blood were taken and the blood sample was stored for host genetics study. The patients with INH resistant TB based on MODS were asked to participate in the INH study. No other tests were conducted on patients with INH sensitive TB. These patients proceeded with standard NTP treatment procedures and were not followed up

beyond 8 months. Standard outcome data (WHO criteria) were collected on all 2,200 patients screened. If the INH resistant TB patients agreed to participate in the INH study, they followed the INH study protocol. Recruitment took two years, followed by 2 years follow-up. Results of treatment completion (8 months) only are reported in this thesis.

Patient information sheets and informed consents (Appendix A and B) in Vietnamese language for both screening and INH study were given to patients. The aims of the study, study method, duration of the study, benefits and discomfort, confidentiality and responsibility were explained in detail to the patients and/or their families. Patients had the right to refuse to participate in the studies or withdraw from the study at any time they wanted without affecting medical care.

2.1.3.1 Inclusion criteria for screening

1. Negative HIV test.
2. Written informed consent.
3. Smear positive pulmonary TB.

2.1.3.2 Exclusion criteria for screening

1. Withhold/unable to consent.
2. Previous TB treatment.
3. HIV infected.
4. Pregnancy.
5. Under 18 years of age.
6. Would receive DOTS outside study centers.

2.1.3.3 Post-screening inclusion criteria

1. Negative HIV test.
2. Written informed consent.
3. Smear positive pulmonary TB.
4. Isoniazid resistant.

2.1.3.4 Post-screening exclusion criteria

1. Withhold/unable to consent.
2. Previous TB treatment.
3. HIV infected.
4. Pregnancy.
5. Under 18 years of age.
6. Would recieve DOTS outside study centers.
7. Isoniazid susceptible.

2.2 Ethics

The INH study protocol was approved by the Institutional Review Board (IRB) at Pham Ngoc Thach Hospital and then by the HCM City Health Services Department. Two individual informed consents (screening study and INH study), which were also approved by IRB of PNT Hospital, were signed by patients enrolled into the respective studies. The study was also approved by the Oxford Tropical Research Ethics Committee (OXTREC) with the code of OXTREC 030 07.

2.3 Case Record File (CRF) (Appendix C)

For screening CRF, data were recorded including study code, study unit, date presenting at admission centre, and date of screening. Other demographic data were obtained such as patient name, age, gender, ethnicity, body weight, occupation, address, duration of illness, date of X ray and X ray result, date of start of treatment, date of initial sputum examination, regimen for treatment, and date of sputum examination after 2 months, after 5 months and after 8 months (completion of treatment) . After 8 months, outcome of completion of treatment was recorded according to NTP guideline (as cured or completed treatment or died or failed or defaulted outcome) . At the screening stage, 2 mls blood for host genetics (for a genome wide-association study, not reported in this thesis) and 2 mls blood for HIV testing were drawn. One sputum sample for MODS testing was sent to PNT Hospital. For patients recruited to the second stage (INH study), the following information was also recorded: symptoms {cough, hemoptysis, fever, sweating, weight loss (over 10% of body weight), chest pain and malaise}, TB contact history, past medical history, social history, medication history, hepatitis evaluation (as risk factors for hepatitis, examination findings) . Any additional drugs taken by patients other than standard DOTS therapy were also recorded during the 8-month treatment regimen. Hepatitis evaluation such as symptoms and liver function tests, and side effects were recorded at month 1, month 2, and at month 3. At month 5 and 8, body weight and symptoms were evaluated.

Sputum culture testing at month 1, month 2, month 5 and month 8 were conducted and the results were recorded in the files. The results of treatment after 8 months were evaluated. Patients were followed up for 16 months more (total for treatment and follow-up is 24 months). At month 12, 18 and 24, patients were invited to come to centres for examination. Body weight and symptoms were recorded, chest radiography and sputum culture tests were conducted. Patients were classified as relapsed based on the criteria from NTP guideline (patient has a bacteriologically confirmed TB disease after he or she was successfully treated during an 8-month previous episode). DOTS administration record, with body weight, types of medication, and doses of medications, were completed in by DTU staff.

In INH study, at month 0, 14 mls blood for examination were taken: 2mls for International Normalized Ratio (INR), 2 mls for urea, electrolytes, creatinine and liver function test (ALT, AST , bilirubin , alkaline phosphate, protein levels and albumin/globulin ratio), 4 mls for serology, 4 mls for viral load and 2 mls for NAT-2 testing. At month 1, month 2 and month 3, only 12 mls blood as mentioned above were taken (because 2 mls blood for NAT-2 were not taken). All results of blood tests were recorded in the file.

Study outline (figure 2.2):

1. Recruit 2200 consecutive HIV negative, primary smear-positive pulmonary TB patients presenting to PNT TB hospital out-patient department and 7 DTUs. Consent for HIV test and isoniazid resistance screening. Consent for genetics study (N=2200) and 2 ml blood stored.
2. Smear positive sputum screened for isoniazid resistance by MODS testing conducted at PNT hospital. We estimated 550 patients with isoniazid resistant TB will be recruited into the study.
3. Enrol isoniazid resistant patients with written informed consent.
4. DNAs were extracted from all isoniazid resistant isolates enrolled into the study and transferred to OUCRU.
5. Isoniazid resistant isolates will be screened for molecular genetic mutation at *katG* 315 or *inhA*-15 by MAS PCR and sequencing at OUCRU. Positive cultures will be genotyped by large sequence polymorphism typing. Minimum inhibitory concentration (MIC) testing for INH at 6 drug concentrations on Lowenstein-Jensen media will be performed at PNT.
6. Obtain 14ml blood sample for host genetic NAT2 testing (OUCRU), liver function test, hepatitis B and C screening and to confirm HIV status (PNT). An aliquot of blood will be stored for hepatitis B and C viral load testing, if serology is positive.
7. Evaluate for hepatotoxicity at month 1, 2 and 3.

8. Record data on: age, gender, HIV status, hepatitis risk factors in addition to socioeconomic and demographic data.
9. Patients will be followed-up through their DTUs or PNT.
10. Patients presenting with symptomatic hepatotoxicity at any time will be managed according to Vietnamese NTP guidelines. Retrospective viral load testing will be done on stored samples from these patients (OUCRU).
11. At 1 month 12 mls blood LFT (additional) (PNT), culture (additional)(PNT).
12. At 2 months 12 mls blood LFT (additional besides routine tests), sputum smear (routine), culture (additional test on routine sample) (PNT).
13. At 3 months 12 mls blood LFT (additional) (PNT).
14. At 5 months, sputum smear (routine), culture (additional test on routine sample).
15. At 8 months, sputum smear (routine), culture (additional test on routine sample). This thesis reports data only to this timepoint.
16. At 12 months: symptomatic evaluation for relapse/failure, sputum smear plus culture if sputum produced.
17. At 18 months symptomatic evaluation for relapse/failure, sputum smear plus culture if sputum produced.
18. At 24 months symptomatic evaluation for relapse/failure, sputum smear plus culture if sputum produced.

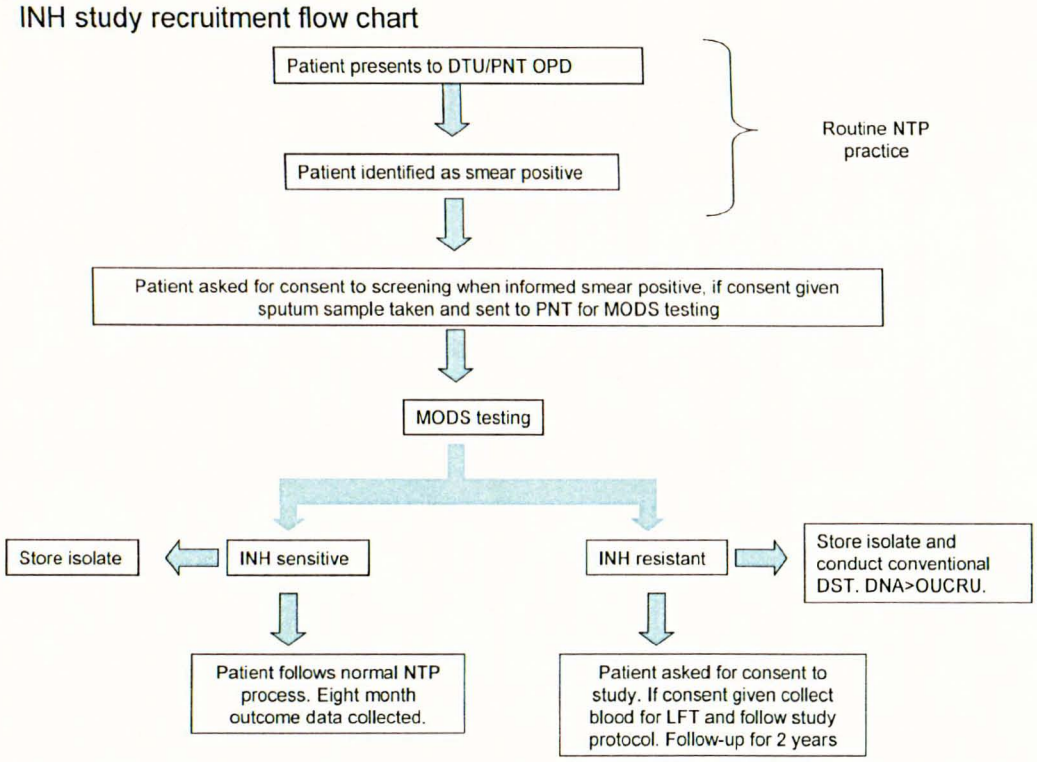


Figure 2-2 : INH study recruitment flow chart

2.4 Smear

The standard operating procedures for smear performed at PNT Hospital and DTUs are based on international guidelines from WHO and CDC USA [65, 69, 319].

Patients with symptoms suggestive of pulmonary TB were requested to provide sputum samples. The patients were asked to inhale deeply 2-3 times, then cough up and spit sputum in the sputum containers which were labeled with the laboratory serial numbers written on it. The sputum should be of good quality with thick, purulent materials and sufficient volumes (about 2-3 ml). The sputum was kept in containers with tightly secure lids and kept away from the sun and heat. The sputum specimens were transported to the laboratory as soon as possible (daily on weekdays and not over 3-4 days from collection).

Slide for smear should be new, unscratched and labeled with laboratory serial number. Smears were made from purulent portion of the sputum using bamboo stick. Sputum was spread on the slide about 2 cm x 3cm in size with good density (not too thick and not too thin). The smear was dried for 15-30 minutes and passed over the flame 3-5 times for 3-4 seconds each time. The smear was then stained by Ziehl Neelsen method (stained with Fuchsin-phenol 0.3%, removed with alcohol-hydrochloric acid 3%, and stained with methylene blue 0.3%).

Microscopy smears were graded and results were recorded in laboratory form and laboratory register book as per Table 2-2 below:

Table 2-2 : Smear microscopy standardized quantitation scale

IUATLD. Technical Guide: sputum examination for tuberculosis by direct microscopy in low income countries. Fifth edition. 2000 **[320]**

2.5 HIV testing

All patients were tested for antibodies to human immunodeficiency virus (HIV) in routine practice. A sample of 3 mls blood from each patient at DTUs was taken and transferred to PNT laboratory for HIV testing according to standard protocols. Samples were tested by ELISA and positive results were confirmed by rapid test and a second ELISA. The results were available after 1 week. There are three tests in routine use including Elisa Genscreen HIV 1/2, rapid test (Biorad, USA) Determine HIV 1/2 (Abott, Japan) and Murex Ag/Ab combination (Murex Diagnostics, USA) to confirm HIV infection. The HIV testing in HCM City is sponsored by the CDC USA.

2.6 MODS

Equipment requirements for MODS are a level 2 Biological Safety Cabinet, inverse light microscope, tissue culture plate, consumables and incubator. MODS is a technique for the rapid detection of *M.tb*. MODS testing was performed by technicians, who were not aware of the clinical diagnosis, at PNT hospital. One sputum sample from smear positive or another sputum sample from each new pulmonary TB patient at units was transferred to laboratory at PNT Hospital for MODS screening. Sputum samples were homogenized and decontaminated by Sputaprep (NaOH-N-Acetyl-L-Ccystein 2%). The processed samples were aliquoted for identification of *M.tb* and DST by MODS.

The MODS method was performed as described in Park *et al.* [321] using the minor modification described by Caws *et al.* [87]. For each processed sample, 2 drug-free

wells (control wells), 1 INH-containing well and 1 RiF-containing well were set up. MODS medium was prepared with 5.9 g Middlebrook 7H9 broth (Difco, Sparks, MD), 3.1 ml glycerol and 1.25 g Bacto casitione (Difco, USA) in 880 ml distilled water. This medium was autoclaved, filtered and stored in 4.5 ml tubes at 4°C. Each new batch of medium was tested for sterility by incubating one aliquot at 37°C for 1 week. Before use, 0.5 ml OADC (oleicacid, albumin, dextrose and catalase), 0.5 ml processed sample and 100 µl PANTA (antibiotic medium supplement) were added into each 4.5 ml tube. Nine hundred microlitres of the suspension was then transferred to each of four wells in a 48 well-plate as described above. Next, 100 µl distilled water was added into the control wells. Finally, 100 µl INH 4 µg/ml (Sigma) or 100 µl RiF 10 µg/ml (Sigma) was added to the INH-containing well and RiF-containing well, respectively. The final concentrations of OADC and PANTA in each well were 10% and 20 µl/ml. The drug concentrations in each well were 0.4 µg/ml for INH and 1 µg/ml for RiF. One susceptible isolate (H37Rv), one INH-resistant clinical isolate and one RiF-resistant clinical isolate were inoculated to the first plate each day. Resistant control isolates are well-characterised clinical isolates from Pham Ngoc Thach laboratory used as routine controls for all DST procedures. A McFarland 0.5 (approximately 10^4 CFU/ml) suspension of each isolate was made and diluted 100-fold (10^2 CFU/ml). A 0.5 ml volume of the final suspension was used as the inoculum. The plate was incubated at 37°C, and the results were recorded on alternate days from day 5 to day 15 and twice a week from day 16 to 1 month. Any cord formation in at least one control well was recorded as a positive MODS culture. If there was any cord formation in both control wells, the drug containing wells were read. If cords were

detected in only one control well, MODS-DST was recorded as uninterpretable for technical analysis. Any isolate with growth in both the control and drug-containing wells was recorded as resistant. If any isolate with growth was observed in control wells but not in the drug-containing wells, a susceptible result was recorded for the relevant drug. Contamination was recorded if there was any growth or turbidity in any negative control well.

Mello *et al.* showed that if INH 0.1 μg/ml was used for the MODS assay, the sensitivity of DST-MODS for detection of INH resistant isolates increased to 96.7% [322]. In this study, INH 0.4 μg/ml was used until September, 2010 when the concentration of INH was reduced to 0.1 μg/ml to increase detection of INH resistant cases.

2.7 Culture, identification and drug susceptibility testing

2.7.1 Culture

All cultures positive by MODS (control wells) were subcultured on LJ medium in duplicate and incubated at 37°C for several weeks. These isolates were then subjected to standard biochemical identification tests, DNA extraction [323] and archiving.

For culture on Lowenstein-Jensen medium performed according to International guidelines [76, 319], the neutralized deposit from MODS were inoculated onto 2 tubes of LJ medium. The LJ tubes were incubated for 72 hours at 36°C-37°C in a horizontal position with the caps loosened. Then, LJ tubes continued to be incubated at 37°C for 8 weeks. The growth of bacteria was noted weekly. The result was released at week 4 and then week 8 (if the results were negative at week 4). The growth of typical TB strains is 'rough, tough and buff'.

2.7.2 Identification

The identification of *M.tb* cultures was performed according to WHO guidelines based on a combination of observed colony morphology and results of biochemical tests including niacin, catalase and nitrate reductase [77].

2.7.3 Drug susceptibility testing

Proportional 1% phenotypic DST method using Lowenstein-Jensen medium was performed at the reference TB laboratory at Pham Ngoc Thach Hospital, which is accredited by the Supranational TB Reference Laboratory Network of Western Pacific Region (Adelaide, Australia) following international and national guidelines [319]. This method determines the resistant proportion of *M.tb* to each drug by comparison of growth rate of *M.tb* on drug-free with drug-containing media. A pure culture of tubercle bacilli in the active phase of growth at 4 weeks was used for the indirect method. A fully susceptible culture of *M.tb* strain H37Rv was used as control strain.

For all four drugs INH, RiF, SM and EMB, the critical concentrations are 0.2 μg/ml, 40 μg/ml, 4 μg/ml and 2 μg/ml respectively with the critical proportion of 1%. The first reading of drug susceptible testing result was done at 28 days of incubation. If the result was negative, the cultures were incubated for a further two weeks and read again at 42 days.

2.8 MIC

INH antibiotic powder (Sigma, Germany) was used for determination of the INH MIC concentration. 20 µg INH powder was dissolved in 10 ml sterile water (solution 1). 1 ml solution 1 was added to 9 ml sterile water (solution 2). The following were INH concentrations for MIC: 0.2 µg/ml (1 ml solution 2), 1µg/ml (0.5 ml solution 1), 2 µg/ml (1ml solution 1), 4 µg/ml (2ml solution 1), 8 µg/ml (4 ml solution 1) and 16 µg/ml (8ml solution 1).

Using the critical concentrations of INH at 0.2 µg/ml, 1 µg/ml, 2 µg/ml, 4 µg/ml, 8 µg/ml and 16 µ/ml and with the critical proportion of 1%, the strain was classified as susceptible at each concentration if the proportion of resistant bacilli was below 1%, and resistant if above 1% .

2.9 DNA extraction

DNA extraction was performed using the CTAB (cetyl trimethylammonium bromide)/ chloroform method after culture on LJ [323]. The purified DNA was dissolved in Tris-EDTA (ethylenediaminetertraacetic acid) buffer, quantified, diluted to the final 15 ng/µl concentration and stored at -20°C.

2.10 MAS_PCR

2.10.1 Multiplex Allele Specific PCR (MAS PCR) to detect *katG* and *inhA* gene mutations

Multiplex allele-specific PCR (MAS-PCR) is an inexpensive method that was developed as an in-house rapid drug resistant assay. MAS-PCR was performed to detect mutations in *katG* (S315T) and *inhA* (C-15T) genes for INH resistance. MAS-

PCR cannot be used to rule out true resistance because approximately 20% of INH resistant isolates will not have mutations in the targeted gene sites.

A MAS-PCR was designed with three primer pairs by Dau Quang Tho [324]. One pair targets *Hsp65* which is specific to *M.tb* and serves as control. A second primer pair targets the inhA promoter region, with the reverse primer designed to detect mutation C-15T in this region. The third primer pair is located inside the *katG* gene where forward primer will yield a PCR product if the isolate is of the wild type.

Each PCR reaction contained 1U Taq DNA polymerase (Bioline London, UK), 0.2 mM each of dNTP (Roche, Lewes, East Sussex, UK) and 150-250 nM of primers and 2 mM $MgCl_2$. As serial dilution of genomic DNA showed that 15 ng of genomic DNA was required to obtain reliable identification of the mutations, 15 ng genomic DNA was added.

All samples were amplified under the same thermocycling conditions: the initial denaturation step at 95°C for 2 minutes, followed by 35 cycles at 95°C for 20 s, 62°C for 20-60 s (60 s for the first 10 cycles, 20 s for the latter 25 cycles), 72°C for 40 s and a final step at 72°C for 5 minutes to complete all reactions. The PCR reactions yielded two bands for wild type isolates, a single band for katG315 mutants and three bands for isolates mutated at inhA C-15T, visualized by 2% agarose gel electrophoresis (20-40 minutes at 140V). The largest band of 656 bp represents the Hsp65 gene of *M.tb*. The second band of 329 bp represents the katG315 wild-type; the isolates without this band have a mutation at *katG*315 and are therefore INH-resistant. The smallest band of 174 bp represents isolates carrying the C-15T mutation in the *inhA* promoter, and are thus INH-resistant.

All primers were designed using Primer Express version 2.0 software (Applied Biosystems Inc., Foster City, CA, USA). The specificity of the primers to *M.tb* was tested in silico and in vitro with Enterobacteriacae Klebsiella spp., K.oxytoca, K. pneumoniae, Escherichia coli, Enterobacter cloacae, Citrobacter freundii, Pantoca spp, and atypical mycobacteria M. avium and M.kansasii.

2.11 Genotype (Large Sequence Polymorphism)

Isolates were typed for large sequence polymorphisms (LSP) at RD105 (East Asian lineage including the Beijing genotype) and RD239 (Indo-Oceanic lineage) to assign major geographical clades. Isolates with neither deletion were assigned to the Euro-American clade on the basis of spoligotyping patterns [128, 325].

RD105: PCR mix was made based on the following components: ELGA water, buffer (w/o $MgCl_2$, EHF), dNTPs, $MgCl_2$, RD105F, RD105R, EHF Taq, DNA template. These components create a volume of 15 µl. PCR programme was set up as follows: 3 minutes denaturation at 95°C, followed by 25 cycles of 30 seconds at 95°C, 15 seconds at 64°C and 4 minutes at 72°C, and a final 6 minutes extension at 72°C. Subsequently, the PCR product was subjected to electrophoresis on a 1% agarose gel in 1X TBE buffer at 120V in 40 minutes. Expected PCR product sizes are positive control – H37Rv of 4kb and deleted product of 850 bp.

RD239: PCR mix was made based on the following components: ELGA water, buffer (w/o $MgCl_2$, EHF), dNTPs, RD239F, RD239R, $MgCl_2$, Taq (bioline), DNA template. These components create a volume of 15 µl. PCR programme was set up as follows: 3 minutes denaturation at 95°C, followed by 25 cycles of 30 seconds at 95°C, 30 seconds at 64°C and 30 seconds at 72°C, and a final 6 minutes extension at 72°C.

Subsequently, the PCR product was subjected to electrophoresis on a 1% agarose gel in 1X TBE buffer at 120V in 40 minutes. Expected PCR product sizes are positive control – H37Rv of 1.8kb and deleted product of 900 bp.

RD150: PCR mix was made based on the following components: ELGA water, EHF buffer with MgCl2 (Roche), dNTPs (Roche), RD150F, RD150R, EHF Taq (Roche), DNA template. These components create a volume of 15 µl. PCR programme was set up as follows: 2 minutes denaturation at 94°C, followed by 30 cycles of 15 seconds at 94°C, 30 seconds at 64°C and 2 minutes and 15 seconds at 68°C, and a final 7 minutes extension at 72°C. Subsequently, the PCR product was subjected to electrophoresis on a 1% agarose gel in 1X TBE buffer at 120V in 40 minutes. Expected PCR product sizes are positive control – H37Rv of 3266 bp and deleted product of 779 bp.

2.12 Host genetics (NAT2)

Illustra ™ DNA Extraction Kit BACC2, (GE Healthcare UK Limited, UK) was used to extract genomic DNA from peripheral blood samples following the manufacturer's protocol. The procedure includes cell lysis, deproteinisation, DNA extraction, DNA precipitation and DNA washing. The NAT-2 genotype was determined by polymerase chain reaction and sequencing PCR of NAT-2. PCR was performed using the primers TGGGCTTAGAGGCTATTT and GAGTTGGGTGATACATACAC in a final volume of 25 µl at the following setting: 4 minutes denaturation at 95°C, followed by 30 cycles of 30 seconds at 95°C, 30 seconds at 58°C and 30 seconds at 72°C, and a final 2 minutes extension at 72°C. Subsequently, the PCR product was sequenced for NAT-2.

The human NAT-2 gene contains 870 nucleotides encoding a protein with 290 amino acids. Nine point mutations have been identified: NAT2-191, NAT2-282, NAT2-341, NAT2-434, NAT2-481, NAT2-590, NAT2-803, NAT2-845, NAT2-857. Recently, a new mutation NAT2-759 was found in a slow acetylator. These mutations occur alone or in combination to yield 14 mutant alleles including NAT2*5A, 5B, 5C, 6A, 6B, 7A, 7B, 12A, 12B, 13, 14A, 14B, 17, 18. The wild-type allele is NAT2*4 and NAT2*5A,5B,5C,6A,6B,7A,7B,14A,14B alleles encode enzymes with lower acetylation activities [326].

A consensus nomenclature for arylamine N-acetyltransferases was set up in 1995. Since that time, many new arylamine N-acetyltransferase alleles have been detected. The first International Workshop for Arylamine N-acetyltransferases was held in 1998 to discuss arylamine N-acetyltransferase gene nomenclature. In this workshop, NAT was designated as the official gene symbol of arylamine N-acetyltransferase. The current nomenclature that is available to the international scientific community can be found at http://nat.mbg.duth.gr

Phenotypes were predicted from genotypes as determined in a study correlating caffeine metabolites with NAT2 genotypes in a healthy volunteer cohort from OUCRU staff (Figure 2.3, thesis of Dau Quang Tho). Polymorphisms at nucleotide number T341C, G590A and G857A were found to define the acetylator phenotype in Vietnamese individuals. Individuals with homozygous mutations in one of these loci or heterozygote at 2 or more loci were slow acetylators.

Figure 2-3: Acetylator status from the AAMU/(AAMU+1X+1U) ratio and the corresponding genotypes deduced from polymorphism at NAT2 allele in 37 healthy Vietnamese volunteers

Bars in the plots show mean and 95% CI of the ratio associated with each genotype.

Differences between the ratio for each NAT2 genotype were calculated pairwise by two-way ANOVA and showed statistical significance, $p<0.001$).

Reproduced from the thesis of Dau Quang Tho.

2.13 Blood tests

2.13.1 International Normalized Ratio (INR)

Two mls blood sample was drawn into a test tube containing liquid sodium citrate as an anticoagulant. The test was done within 4 hours of taking the blood. The volume and the quality of the blood sample were checked. The blood was centrifuged at a speed of 2,000-3,000 cycles for 10 minutes to separate blood cell from plasma. The standardized volume of plasma was taken into Stant4 machine from Japan for this procedure, which yields results in about 5 to 10 minutes.

2.13.2 Urea, creatinine, ALT, AST, bilirubin, alkaline phosphate, protein, A/G, electrolytes

Two mls blood sample was drawn into a test tube containing liquid lithium heparin . For urea, creatinine, ALT, AST, bilirubine, alkaline phosphate, protein and A/G, the volume and quality of the blood sample were checked. The blood was centrifuged at a speed of 2,000-3,000 cycles for 10 minutes to separate blood cells from plasma. The standardized volume of plasma for each test was introduced into a Hitachi911 autoanalyser (Boehringer Mannheim, USA) for this procedure, which yields results in about 5-10 minutes. For electrolytes, the plasma volume was put into AVL9181 for this procedure, which yields results in about 2-5 minutes.

2.13.3 HBV and anti HCV serology

Four mls blood was drawn into aa anti-coagulant tube. The volume and the quality of the blood sample were checked. The blood was centrifuged at a speed of 2,000-3,000 cycles for 10 minutes to separate blood cells from plasma. Rapid tests for HCV (Acon Laboratories, USA) and for HBV (Determine HBsAg Alere, USA) were conducted, yielding results in about 5 to 10 minutes.

2.14 Statistics

Demographic characteristics were analysed by calculating mean and range for normally distributed continuous variables and median and interquartile range (IQR) for other continuous variables. Categorical variables were described by frequency. Treatment results were categorized according to the WHO classification scheme as well as Vietnamese NTP guidelines as cured, treatment completed, died, treatment failure, lost to follow-up or not evaluated. For further analysis treatment outcomes were reclassified into favourable (cured and treatment completed) or unfavourable (died, treatment failure, lost to follow-up or not evaluated). Variables associated with unfavourable outcome were assessed by univariate or multivariate logistic regression analysis.

The cumulative incidence (risk) and incidence rate of drug induced hepatotoxicity were calculated. The risk factors for ATDIH were also assessed by univariate and multivariate logistic regression. The correlation between HBV DNA and ALT values was calculated using Pearson's correlation. All analyses were done by using R

software. The odds ratio with 95% confidence interval (CI) was used to assess the strength of associations with $p<0.05$.

Chapter 3

Isoniazid resistant tuberculosis and treatment outcomes

3.1 Aims

1) To evaluate treatment outcomes in patients treated according to Vietnam National Tuberculosis Programme guidelines.

2) To compare treatment outcomes of two standardised regimens (2SRHZ/6HE and 2RHZE/6HE) used in routine practice.

3) To investigate the demographic, clinical, paraclinical characteristics and treatment outcomes of patients with INH resistance confirmed by LJ cuture and DST results:

3.2 Introduction

While there is a pressing need to prevent multidrug resistance (MDR) the most effective regimen for the treatment of isoniazid-resistant TB is not known"

(WHO Treatment of tuberculosis Guidelines 4th Edition, 2009) [265]

According to the WHO, drug-resistant TB exists in all countries in the world and is a major concern in several countries. Globally, WHO estimated 3.7% (95% CI 2.1-5.2%) of TB cases were MDR-TB in 2011. In Vietnam, the percentage of MDR TB among new TB cases was estimated to be 2.7% (95% CI 2.0-3.6%) in 2011 [327]. Among drug-resistant TB strains worldwide, isoniazid resistant TB is the most common. In Vietnam, streptomycin resistance is the most common, followed by INH

resistance. Data reported to the World Health Organization from 1994 to 2009 showed the percentage of INH resistant (INH^R) among new TB cases was very high in the Eastern European region (33.5%, 95% CI: 24.8%- 42.2%). Meanwhile, Vietnam and other countries including China, the Dominican Republic and parts of India reported an INH^R TB rate between 15% and 20% [328] (figure 3.1). There have been few studies on INH^R TB which is not MDR. INH resistance in *M.tb* will reduce the success rate of TB treatment, fuel the generation of new MDR-TB strains and reduce the effectiveness of INH prophylaxis.

Figure 3-1 : World map showing the percentage of incident TB cases with isoniazid resistance (INH-R) from the most recent survey in each setting in three time periods : (a) 1994-1999, (b) 2000-2004, (c) 2005-2009.

Reproduced from Jenkins et al., PLos ONE 2011 [328].

Currently, due to a lack of laboratory capacity in high burden settings, most cases of drug resistant TB in newly presenting patients are unidentified and are treated with the standard first line regimen (2HRZE/4HR). Outcomes using this regimen for patients infected with undiagnosed MDR TB are extremely poor [329]. For cases which are isoniazid resistant but remain sensitive to rifampicin, outcomes are also known to be worse than for fully sensitive TB although estimates of the relative risk in meta-analyses are imprecise due to inconsistencies in treatment regimens, drug susceptibility testing (DST) methodology and reporting of results across studies [330]. A study conducted in the Western Cape Province, South Africa showed 16% of patients with INH^R TB had poor outcomes among whom 61% developed MDR-TB [331]. In contrast, other studies reported that there were no differences in treatment outcomes in these two groups. The later studies showed no differences because INH resistance was detected early and modified regimens were used. INH^R TB should be quantified and detected early because treatment outcome will be improved when the duration of treatment is prolonged and the TB treatment regimen is adjusted [332, 333].

In Ho Chi Minh City, 25% of primary patients and 54% of retreatment patients were found to have isoniazid (INH) resistant TB in 2006 [318]. The key drug in the first days of therapy, INH kills the largest population of *M.tb* bacteria in log-phase growth within the first 2-3 days of therapy.

Isoniazid resistance is associated with a poor outcome and the development of MDR TB on treatment in Vietnam [318]. Within the NTP framework all new patients receive a DOTS regimen of 2 months streptomycin (SM), izoniazid (INH), rifampicin

(RiF) and pyrazinamide (PZA) followed by 6 months of isoniazid and ethambutol (EMB) (2SHRZ/6HE). Since October 2009, SM has been replaced by EMB in the regimen for newly diagnosed pulmonary TB patients in Ho Chi Minh City. The standard regimen for these patients is now 2RHZE/6HE. Upon treatment failure or relapse, drug susceptibility testing is begun and a retreatment regimen with the same drugs is commenced (2 months SHRZE, 1 month HRZE and 5 months $H_3R_3E_3$). This regimen showed just 33% successful sputum smear conversion among MDR cases in a retrospective analysis [318]. The subsequent relapse rate was not determined. Green light committee procured second line drugs have been available in a limited number of NTP centres since 2010 through the programmatic management of MDR-TB (PMDT).

Also within the framework of the NTP, all TB patients are tested for HIV and evaluated for sputum smear positivity at 2 months, 5 months and 8 months (completion of treatment). Currently, a smear-positive result at 2 months does not lead to a change in drug therapy. Patients in the INH^R study were also additionally evaluated for culture positivity by LJ at months 5 and 8 and followed for relapse at 12, 18 months and 24 months by sputum smear microscopy and culture. Treatment outcomes were evaluated according to Vietnam NTP for all patients in the screening study. Treatment outcomes for patients with INH^R and MDR TB diagnosed by LJ DST (gold standard) were evaluated. The additional failure case detection with the addition of culture to the definition was also determined.

3.3 Results

3.3.1 Demographic characteristics of patients in screening study

During the study period between 1 December 2008 and 30 June 2011, at eight district TB units (including districts 1, 4, 6 and 8, Phu Nhuan, Binh Thanh and Tan Binh districts and PNT out- patient department), there were 5,036 cases diagnosed as new smear positive pulmonary TB including HIV infected individuals. 2,190 (43.5%) of these cases met eligibility criteria and agreed to enter the screening study. District 3 (with 9 cases) withdrew from the study due to low recruitment. Seventy- nine further cases were diagnosed with HIV infection post-recruitment and 11 cases were aged < 18 years old (from 13 to 17 years old) including 7 males and 4 females (1 case each from district 1, district 6, Tan Binh district, 2 cases from district 4, 3 cases each from district 8 and Phu Nhuan district) and were therefore excluded. Finally, there were 2,091 cases eligible and included in the screening study. The recruitment flow-chart is shown in Figure 3-2. The demographic characteristics for these patients are presented in Table 3-1 and occupation distribution in Figure 3-3.

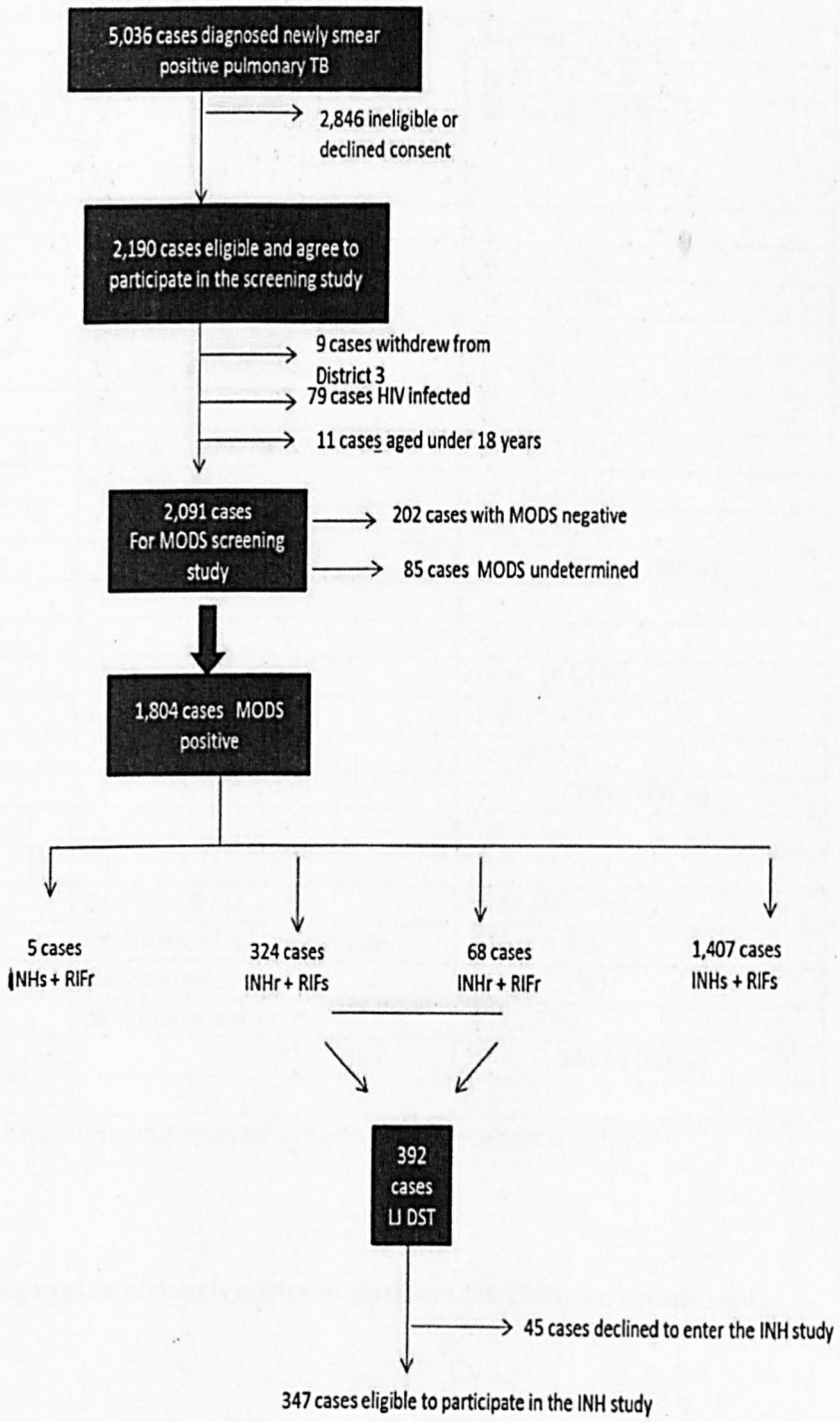


Figure 3-2 : Recruitment flowchart for INH study

Characteristics		Summary measures n (%)
Age (years)		Median 39 [range 18-92]
Male gender		1541 (73.7%)
Duration of illness (day)* Median [range]		30 days [1-365]
Districts		
	Pham Ngoc Thach	127 (6.0%)
	Phu Nhuan	334 (16.0%)
	District 6	502 (24.0%)
	District 4	155 (7.4%)
	Binh Thanh	278 (13.3%)
	District 1	231 (11.0%)
	District 8	259 (12.4%)
	Tan Binh	205 (9.8%)
Total		**2091 (100%)**
Ethnicity		
	Kinh	1968 (94.1%)
	Chinese	108 (5.1%)
	Other	15 (0.8%)
Total		**2091 (100%)**
Occupation		
	Manual labour	1457 (69.7 %)
	Professional/administrative	224 (10.7%)
	Retired/housewife	362 (17.3%)
	No employment	48 (2.3%)
Total		**2091 (100%)**

* 10 patients did not recall the duration of symptoms since onset of illness

Table 3-1 : Demographic characteristics of patients for INH screening study

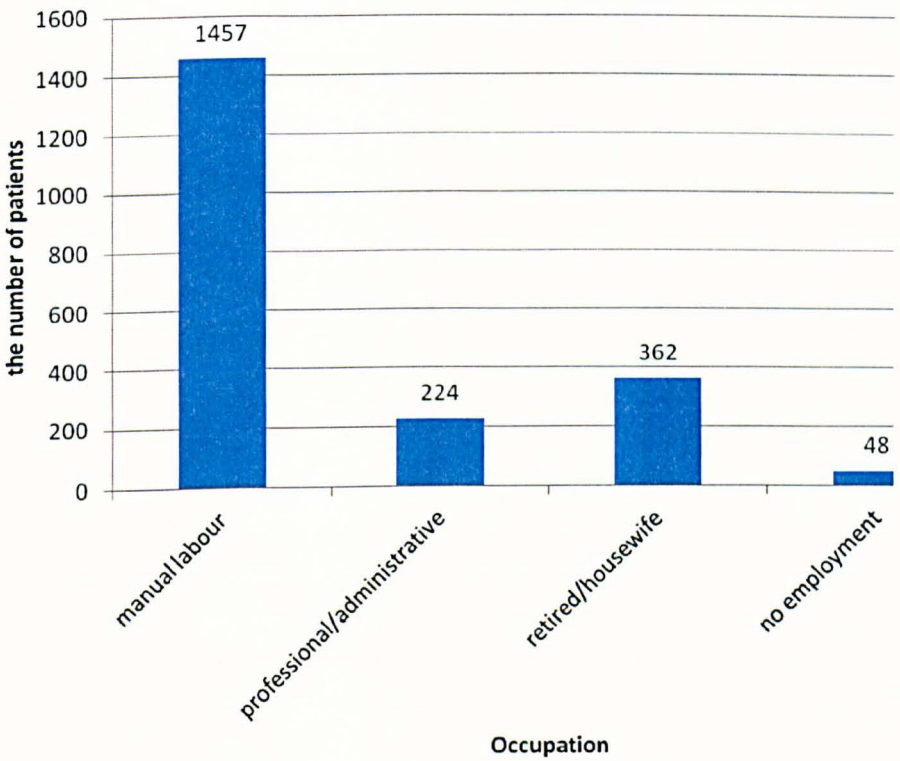


Figure 3-3 : Occupation of all patients in the screening study

Ninety percent of patients with new smear positive pulmonary TB were under 58 years of age. Table 3-2 and Figure 3-4 shows the age groups and gender among the screening patients.

Age group (years)	No. of cases n (%)	Male n (%)	Female n (%)
18 - 27	511 (24.4)	340 (22.1)	171 (31.1)
28 - 37	463 (22.1)	318 (20.6)	145 (26.4)
38 - 47	500 (23.9)	419 (27.2)	81 (14.7)
48 - 57	427 (20.4)	325 (15.5)	102 (18.5)
58 - 67	115 (5.5)	90 (4.3)	25 (4.5)
68 - 77	54 (2.6)	37 (1.8)	17 (3.1)
≥ 78	21 (1.0)	12 (0.5)	9 (1.6)
Total	2091 (100)	1541 (100)	550 (100)

Table 3-2 : Newly smear positive pulmonary TB patients by age and gender in screening study

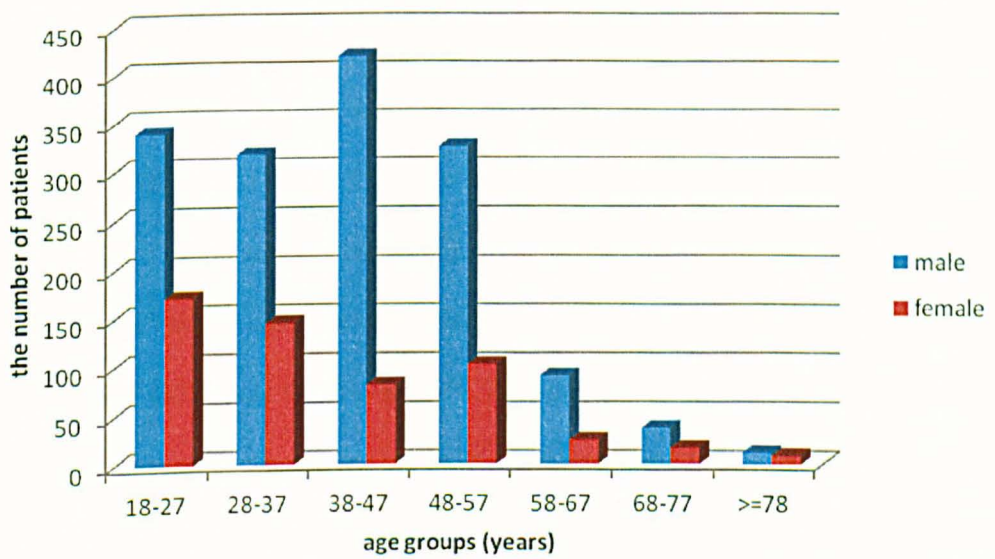


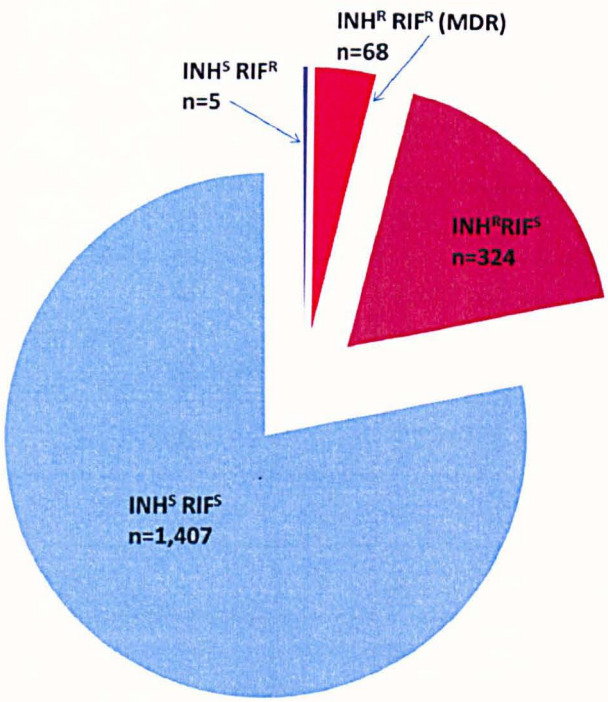
Figure 3-4 : Newly diagnosed smear positive pulmonary TB patients by age and gender in the screening study

3.3.2 MODS results

Sputum samples from 2,091 new smear positive pulmonary TB patients were sent for MODS testing. Susceptibility to RiF and INH by MODS was determined. MODS cultures were positive for mycobacteria growth in 1,804 cases. Of these, 1,407 cases (67.3%) were susceptible to both drugs, 324 cases (15.5%) were resistant to INH and susceptible to RiF, 5 cases (0.2%) were susceptible to INH and resistant to RiF, 68 cases (3.3%) were resistant to both INH and RiF (MDR) (Figure 3-5). There were 202 cases (9.7 %) which had a negative MODS culture and 85 cases (4.1%) that were indeterminate. The indeterminate category included isolates for which there was a negative result in the MODS control well and/or contamination of the control or sample well.

The median time to culture positivity for MODS was 10 days (range 4-60 days). The critical concentration for INH susceptibility testing from the beginning of the study (December 2008) to 31 August 2010 was 0.4 μg/ml and from the 1st of September 2010 to the end of the study (June 2011), the concentration was changed to 0.1 μg/ml. In a systematic review and meta-analysis of the MODS assay, with a 0.4 μg/ml cutoff, sensitivity for INH^R was 90.0% (84.5-93.7) compared to LJ DST. With a 0.1 μg/ml critical concentration, sensitivity for INH^R compared to LJ DST increased to 97.7% (94.4-99.1) [84]. In the present study, the change of INH critical concentration increased the percentage of isolates determined as INH^R by MODS from 17.4% to 21.2%. The overall MODS results are presented in Table 3-3 and Figure 3-5.

The percentage of isolates resistant to INH by MODS which were confirmed by LJ DST decreased significantly following the concentration change (89.7%, n=234/261) for 0.4µg/ml and 81.75 % (n=112/137) for 0.1µg/ml. P=0.026.



RIF^{R}: Rifampicin resistant,

INH^{R}: Isoniazid resistant

INH^{S}: Isoniazid susceptibile

RIF^{S}: Rifampicin susceptible

Figure 3-5 : Drug Resistance profile of 1,804 isolates positive by MODS

MODS result	Susceptibility pattern	Number of cases n (%)
Positive MODS culture		
	$INH^R + RIF^R$	68 (3.3)
	$INH^R + RIF^S$	324 (15.5)
	$INH^S + RIF^S$	1,407 (67.3)
	$INH^S + RIF^R$	5 (0.2)
	Total	1,804 (86.3)
Negative MODS culture		202 (9.7)
Undetermined MODS		85 (4.1)
Total		**2,091 (100%)**

RiF^R:Rifampicin resistant,

INH^R: Isoniazid resistant

INH^S: Isoniazid susceptibile

RiF^S: Rifampicin susceptible

Table 3-3 : MODS culture results of 2,091 patients in screening study

There were 392 cases with INH^R by MODS (324 cases which were INH^R + RIF^S and 68 cases which were MDR: INH^R + RIF^R). Of 392 cases with INH resistant TB (+/- RIF resistance) by MODS, 45 **(11.5%)** cases declined to participate in the INH study. Finally, 347 cases were eligible to enter the INH study. The verbally reported reasons for not participating in the study were unstable residence, old age, concomitant illnesses such as diabetes or hypertension, a fear of giving blood, transfer to private clinics for treatment, being busy, work place far from DTUs, and long follow-up time (24 months).

Finally, 347 cases were eligible and consented to enter the INH main study with the numbers recruited in each district shown in Table 3-4:

District	Number of cases (%)
District 1	48 (13.8%)
District 4	28 (8.1%)
District 6	73 (21.0%)
District 8	39 (11.2%)
Phu Nhuan	53 (15.3%)
Binh Thanh	58 (16.7%)
Tan Binh	27 (7.8%)
Pham Ngoc Thach	21 (6.1%)
Total	347 cases (100%)

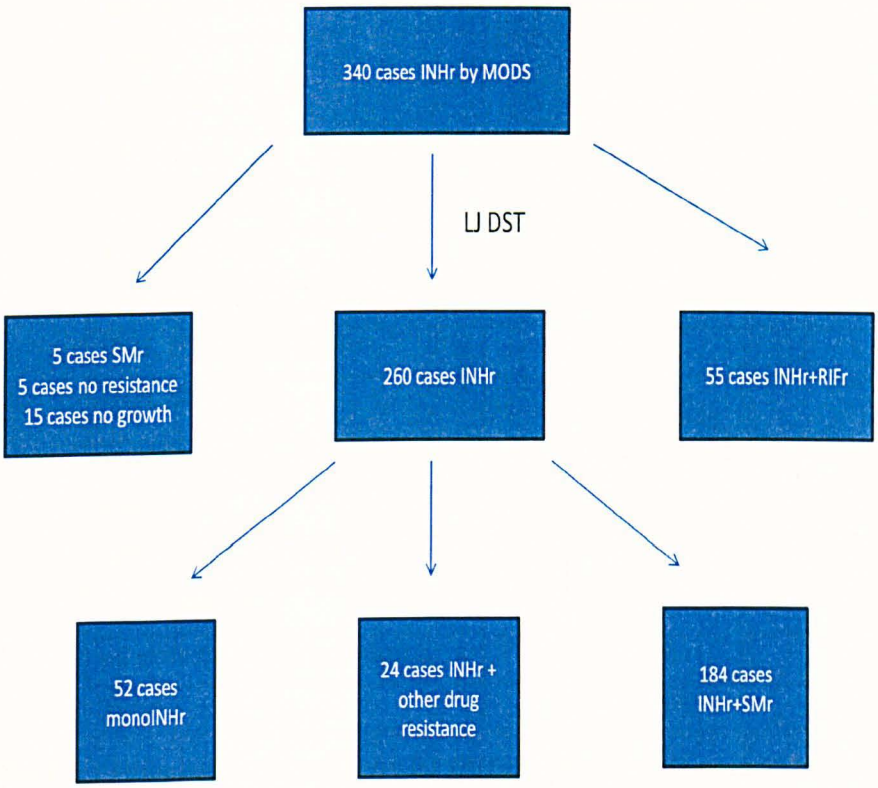
Table 3-4 : The number of participants with INH^R (+/- RIF^R) by MODS in each district participating in the INH resistant TB study

3.3.3 LJ Culture and DST results

347 samples from 347 patients with INH resistance (+/- RiF resistance) by MODS were sent for Drug Susceptibility Testing on Lowenstein Jensen media. Seven samples were irretrievable. Susceptibility was determined for INH, RiF, SM, EMB and PZA.

Overall, 315/340 (92.6%) isolates INH resistant by MODS were confirmed INH resistant by LJ DST. Fifty-two cases (15.3%) were INH monoresistant and 184 cases (54.1%) were INH and SM resistant and 24 (7.1%) cases with other patterns of INH polyresistance (figure 3.6).

There were 55 MDR cases with additional resistance to other drugs. There were no cases with only INH and RiF resistance (Table 3-5 and Figure 3-7). Further analysis of INH-resistant TB and MDR TB cases was based upon those confirmed by LJ DST.



INH^R= Isoniazid resistant

SM^R= streptomycin resistant

RIF^R= rifampicin resistant

Figure 3-6: LJ DST results of 340 cases with INH^R by MODS

Resistance profile by LJ DST	The number of casesn (%)
INH	52 (15.3)
INH, SM	184 (54.1)
INH, PZA	2 (0.6)
INH,SM,EMB	4 (1.2)
INH,SM, PZA	17 (5.0)
INH, SM, EMB, PZA, RIF	7 (2.1)
INH, SM, RIF, EMB	17 (5.0)
INH, SM, RIF	22 (6.5)
INH,SM, PZA, RIF	9 (2.6)
SM	5 (1.5)
SM, INH, EMB,PZA	1 (0.3)
no resistance	5 (1.5)
no growth or contamination	15 (4.4)
Total	340 (100)

Table 3-5 : Drug resistant profile by LJ DST of 340 cases detected as INH^R by MODS

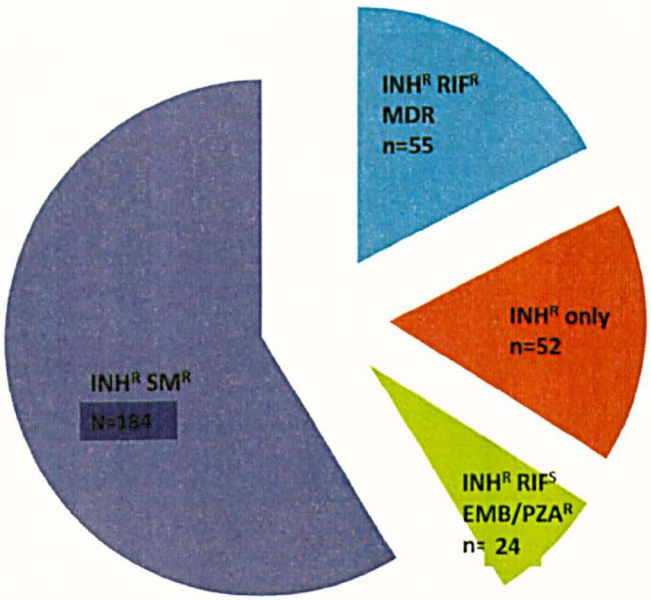


Figure 3-7: Distribution of drug susceptibility profiles for 315 isolates resistant to INH by LJ DST

3.3.4 Demographic risk factors for INH resistant TB and MDR TB

Demographic risk factors age, gender and district were analysed for association with both INH resistant (non-MDR) TB or MDR TB. Results are shown in Table 3-6. There were no significant associations of gender or district with either INH-resistant or MDR TB. Age 26-35 years was significantly associated with MDR TB in comparison with 18-25 year olds as baseline (OR=2.98 [95% CI 1.26-7.03], P=0.013). There was no significant association between age and INH^R TB.

	MDR TB			INH RESISTANT TB		
	OR	95% CI	P-value	OR	95% CI	P-value
Male sex	1.00	0.54-1.85	0.993	1.05	0.80-1.38	0.710
Kinh ethnicity	1.10	0.34-3.59	0.870	1.35	0.78-2.35	0.282
Age category						
1: 18-25 years	BASELINE					
2: 26-35 years	**2.98**	**1.26-7.03**	**0.013**	1.11	0.77-1.59	0.584
3: 36-45 years	1.38	0.54-3.55	0.499	1.07	0.75-1.53	0.715
4: 46-55 years-	1.26	0.47-3.33	0.648	0.79	0.54-1.16	0.229
5: ≥56 years	0.74	0.19-2.89	0.665	0.77	0.48-1.21	0.255
District						
Pham Ngoc Thach	BASELINE					
Phu Nhuan	1.06	0.28-4.06	0.936	0.94	0.53-1.68	0.838
District 6	0.81	0.21-3.03	0.751	0.82	0.47-1.43	0.492
District 4	0.81	0.16-4.11	0.803	1.18	0.63-2.25	0.598
Binh Thanh	1.76	0.48-6.44	0.393	1.39	0.79-2.46	0.259
District 1	1.97	0.53-7.33	0.309	1.33	0.	0.348
District 8	1.42	0.37-5.45	0,611	0.82	0.44-1.50	0.511
Tan Binh	0.60	0.12-3.02	0,535	0.76	0.40-1.45	0.405

Table 3-6: Demographic risk factors for MDR and INH resistant TB

3.3.5 Treatment outcomes in screening patients

One patient CRF was irretrievable and therefore 2,090 new pulmonary TB patients were evaluated by sputum smear microscopy after 5 and 8 months according to WHO guidelines followed by Vietnam NTP. The majority of patients (95.1%, n=1987/2090) received the daily regimen recommended by the Vietnam NTP. Prior to October 2009 this regimen was SM, RiF, INH and PZA for the first two months (intensive phase) followed by 6 months with INH and EMB (continuation phase) (2SRHZ/6HE). In October 2009 there was a change to the standard national treatment regimen; SM was replaced by EMB (2RHZE/6HE) which enabled us to compare treatment outcomes on the two regimens.

In total, 547 patients recieved 2SRHZ/6HE and 1,440 patients recieved 2RHZE/6HE. The remaining 103 patients received alternative individualized regimens, such as 2SRHZ/RHZ/5HE, 2SRHZE/RHZE/5RHE, 3RHZE/5HE, 3RHZE/3RH, 2SRHZ/4RH and 2RHZE/4RH.

Patients treated at PNT out-patient department were much more likely to receive individualized treatment regimens than those treated at the district TB units. Of those receiving individualized regimens, 95/103 (92.2%) were treated at PNT out-patient department.

Table 3-7 and Figure 3-8 show the overall WHO defined treatment outcomes of the patients in the screening study. 1,858 (88.9%) patients were cured and 6 (0.3%) patients were classified as 'treatment completed' and so 1,864 (6+1858) patients (89.2%) were classified as having a favorable outcome. 16 patients died (0.8%), 121

(5.8%) treatment failed, 82 were lost to follow up (3.9%) and 7 (0.3%) were not evaluated. Therefore, a total of 226/2,090 patients (10.8%) were classified as having an unfavourable outcome by WHO criteria.

	Treatment outcomes	Number of cases n (%)
Favourable		
	Cured	1858 (88.9)
	Treatment completed	6 (0.3)
Unfavourable		
	Died	16 (0.8)
	Treatment failed	121 (5.8)
	Lost to follow-up	82 (3.9)
	Not evaluated	7 (0.3)
	Total	**2,090 (100)**

Table 3-7: Treatment outcomes of patients in the screening study by WHO definitions including smear results only

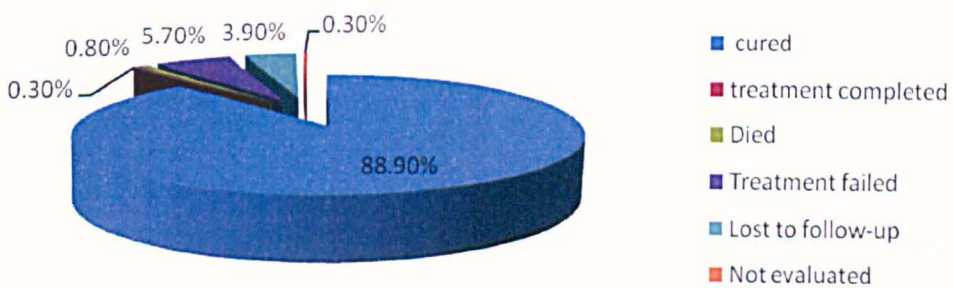


Figure 3-8 : Treatment outcomes in patients for screninng study by WHO definitions including smear results only.

3.3.6 Comparision of treatment outcomes of two standardized treatment regimens (2SRHZ/6HE and 2RHZE/6HE)

Treatment outcomes were compared between the two standardized regimens (2SRHZ/6HE and 2RHZE/6HE) in patients within the screening study (Table 3-8). The crude OR for unfavourable outcome was 0.97 [95% CI 0.70-1.34], P=0.871 for patients receiving 2RHZE/6HE compared to those receiving 2SRHZ/6HE. Adjusting for diagnosed drug resistance did not change the OR: adjusted OR= 0.98 [95% CI 0.67- 1.42], P=0.912 (Table 3-9).

Outcome	Regimen	
	2SRHZ/6HE n (%)	2RHZE/6HE n (%)
Cured	491 (90.1)	1287 (89.4)
Treatment completed	0	2 (0.1)
Died	4 (0.7)	11 (0.7)
Treatment failed	27 (4.5)	88 (6.0)
Lost to follow-up	24 (4.3)	46 (3.1)
Not evaluated	1 (0.1)	6 (0.4)
Total	547 cases (100%)	1,440 cases (100%)

Table 3-8: Treatment outcomes in screening patients with two main regimens

	Favourable outcome n (%)	Unfavourable outcome n (%)	Crude OR [95%CI] and p value*	Adjusted OR [95%CI] p value**
2SRHZ/6HE (n=547)	491 (89.8)	56 (10.2)	Baseline	
Regimen 2RHZE/6HE (n=1,440)	1289 (89.5)	151 (10.5)	0.97 [0.70-1.34] P=0.871	0.98 [0.67- 1.42], P=0.912
Total (N=1987)	1780	207		

*univariate and multivariate logistic regression
**adjusted for MDR, SM and INH resistance

Table 3-9: Comparison of treatment outcomes by treatment regimen adjusted for drug resistance

3.3.7 Risk of unfavourable WHO outcome by drug resistance pattern

Overall, patients with INH resistant TB confirmed by LJ DST did not have a higher risk of unfavourable WHO outcome on standard treatment regimens (OR= 1.13 [95% CI 0.73-1.77], P=0.582). Patients with MDR TB had a much greater risk of unfavourable outcome (OR=11.61 [95% CI 6.58-20.6], P<0.001) (Table 3-10).

Resistance	Unfavourable outcomes n/N (%)	OR	95% CI	P-value
INH +RiF susceptible	154/1690 (9.1%)	BASELINE		
INH resistant	25/245 (10.2%)	1.13	0.73-1.77	0.582
MDR	28/52 (53.8%)	11.64	6.58-20.6	<0.0001

Excluding patients receiving individualized regimens.

Table 3-10: Unfavourable WHO treatment outcomes by drug resistance profile group

3.3.8 Demographic, clinical, and paraclinical characteristics of patients with INH resistant TB

Of 347 cases resistant to INH by MODS (+/- resistance to RiF), LJ DST for INH, RiF, SM, PZA and EMB was performed for 340 cases (7 cases were irretrievable for LJ culture and DST).

The results showed 5 cases with SM monoresistance, 5 cases susceptible to all drugs tested and 15 cases with no growth on LJ. The remaining 315 cases included 55 cases with both INH and RiF resistance (MDR TB +/- resistance to other drugs). One MDR isolate was identified as Non Tuberculosis Mycobacteria (NTM) and therefore the case was excluded. Fifty-four cases were therefore classified as MDR TB cases for further analyses. The remaining 260 cases were resistant to INH and susceptible to RiF but +/-resistance to other drugs (SM, PZA and EMB) by LJ. These cases were classified as INH-resistant (non MDR) group for further analyses. These 260 cases included 52 cases with INH monoresistance, 184 cases with INH^R and SM^R, and 24 cases with other susceptibility patterns including INH^R (Figure 3-6).

Demographic chacteristics were therefore analysed for 314 cases (Table 3-11). The median age was 38 years (range 18-91), 230 (73.2%) were male. The median duration of illness was 30 days (IQR: 14-60). Three quarters of the patients were manual labourers (72.6%, n=228/314) (Table 3-11).

Characteristic	n (%)
Age	median: 38, [range 18-91 years]
Male gender	230 (73.2%)
Occupation	
Manual labour	228 (72.6%)
Professional/administrative	30 (9.6%)
Retired/housewife	47 (15.0%)
No employment	9 (2.9%)
Total	314 (100%)
Duration of illness	median : 30 days, range [2-365 days]

Table 3-11: Demographic characteristics of 314 cases with INH^r (+/- RIF^r) confirmed by LJ culture

Table 3.12 and Figure 3-9 show the distribution of age group and gender in patients with INH^R (+/- RiF^R) confirmed by LJ DST.

A total of 260 cases with INH^R (not MDR) diagnosed by LJ DST were analysed. Baseline demographics for these patients are shown in Table 3-13. Age and gender distribution of 260 patients with INH^R (not MDR) were showed in Table 3-14 and Figure 3-10.

Age group (years)	Number of cases n (%)	Male n (%)	Female n (%)
18 - 27	80 (25.5)	48 (20.9)	32 (38.1)
28 - 37	74 (23.6)	55 (23.9)	19 (22.6)
38 - 47	86 (27.4)	74 (32.2)	12 (14.3)
48 - 57	50 (15.9)	36 (15.7)	14 (16.7)
58 - 67	18 (5.7)	13 (5.7)	5 (6.0)
68 - 77	4 (1.3)	3 (1.3)	1 (1.2)
>= 78	2 (0.6)	1 (0.4)	1 (1.2)
Total	314 (100)	230 (100)	84 (100)

Table 3-12: Patients with INH resistance (+/- RIF^r) diagnosed by LJ by age and sex

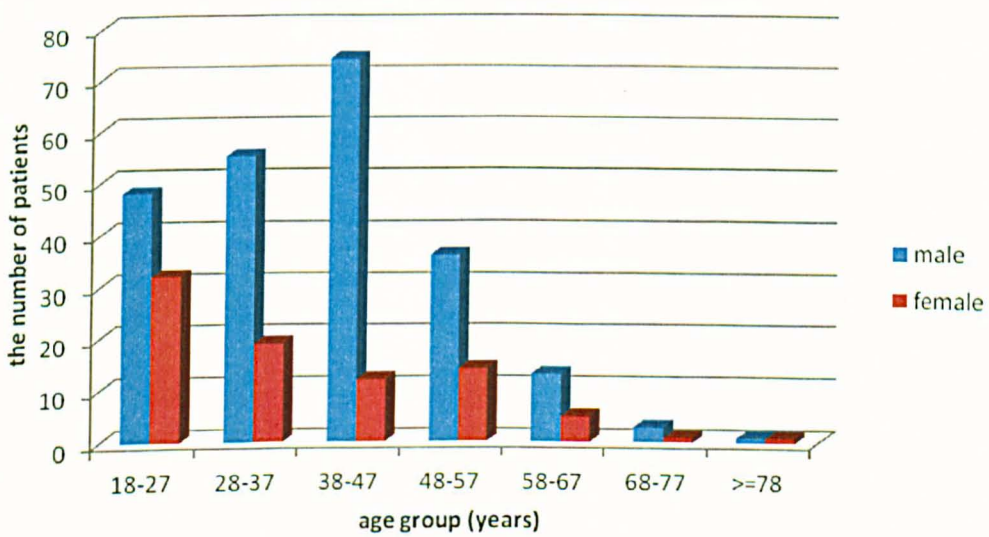


Figure 3-9 : Patients with INH^R (+/- RIF^R) diagnosed by LJ culture by age and gender

Characteristic	n (%)/median [IQR]
Age	39 years [27-48]
Male gender	189 (72.7%)
Occupation	
Manual Labour	183 (70.4%
Professional/administrative	24 (9.2%)
Retired/housewive	45 (17.3%)
No employment	8 (3.1%)
Total	260 (100%)
Duration of illness	30 days, [14-60]

Table 3-13: Demographic characteristics of 260 patients with INH^r (not MDR) by LJ DST

Age group (years)	Number of cases (%)	Male n (%)	Female n (%)
18 - 27	70 (26.9)	41 (21.7)	29 (40.8)
28 - 37	55 (21.2)	39 (20.6)	16 (2.3)
38 - 47	69 (26.5)	61 (16.9)	8 (11.3)
48 - 57	44 (16.9)	32 (16.9)	12 (16.9)
58 - 67	16 (6.2)	12 (6.3)	4 (5.6)
68 - 77	4 (1.5)	3 (1.6)	1 (1.3)
>= 78	2 (0.8)	1 (0.5)	1 (1.3)
total	260 (100)	189 (100)	71 (100)

Table 3-14: Patients with INH^R (not MDR) confirmed by LJ DST by age and gender

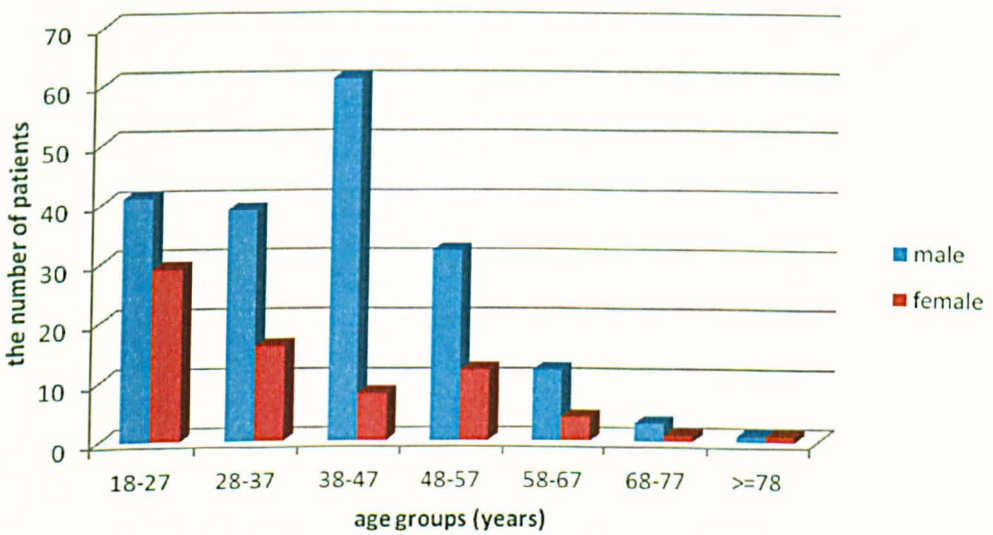


Figure 3-10 : Age and gender distribution of 260 patients with INH^R (not MDR) confirmed by LJ DST.

For 260 patients with INH^R diagnosed by LJ culture, symptoms were assessed. The results showed 93.4% with cough,17.8% with haemoptysis ,47.7% with fever, 18.2% with night sweat , 26% with weight loss, 29.5% with chest pain and 36% with malaise (Figure 3-11).

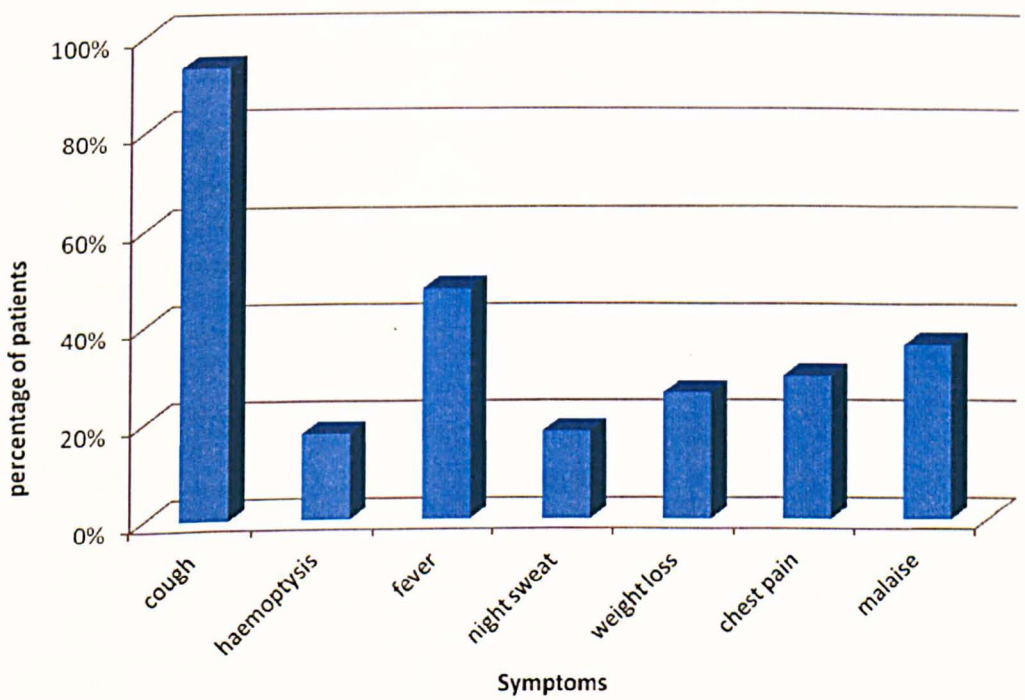


Figure 3-11 : Symptoms in patients with INH^R (not MDR) diagnosed by LJ culture.

In 260 cases with INH^R diagnosed by LJ cuture, blood biochemistry was assessed in 252 cases for INR, PT, urea, electrolytes (Na, K, Ca, Cl), creatinine, ALT, AST, total bilirubin, direct bilirubin, indirect bilirubin, AP, protein and albumin/globumin ratio. The results are shown in Table 3-15.

Blood tests	normal values	number evaluated	mean (SD)	range (lowest-higest)
INR	2-3	238	1.2 (0.5)	0.8-5.6
PT	70-100%	126	83.8 (14.5)	21-100
Urea	<8.3mmol/l	252	4.2 (1.6)	1-11.5
Na	135-145 mmol/l	252	138.6 (7.1)	143-150
K	3.5-5.1 mmol/l	252	3.9 (0.9)	2.7-10.3
Ca	2.2-2.5 mmol/l	252	2.2 (0.2)	1.2-2.9
Cl	95-110 mmol/l	252	100.9 (3.4)	90-110
Creatinine	55-110 µmol/l	252	85.9 (45.5)	58-778
AST	9-48 U/l	252	30.4 (39.4)	6-501
ALT	5-45 U/l	252	30.3 (48.8)	5-521
Total Bilirubin	≤19µmol/l	252	14.4 (6)	1.1-52.6
Direct Bilirubin	≤5.1µmol/l	252	7.5 (50.2)	0.7-801
Indirect Bilirubin	≤13.9µmol/l	252	10.1 (4.4)	1-41.8
AP	<258 U/l	252	182.1 (60.4)	93-451
Protein	60-80g/l	252	87.3 (52.4)	70-908
albumin/globulin ratio	1.3-1.8	252	1.2 (0.3)	0.3-2.4

Table 3-15: Blood biochemistry in patients with INH^R confirmed by LJ DST

3.3.9 Treatment outcomes of patients with INH resistant TB and MDR TB

Two groups were defined by LJ DST: MDR and INH^R (non MDR) TB.

MDR TB, INH^R + RiF^R (group 1) including 54 patients, excluding one NTM isolate. There were 54 MDR TB patients who were evaluated at month 8 (treatment completion). Based on WHO criteria the treatment results were: 25 cases (46.3%) cured, 27 cases (50%) treatment failed, 1 case (1.9%) lost to follow-up and 1 case (1.9%) was not evaluated.

Patients with INH^R, not MDR TB (group 2) included 260 cases that were evaluated at month 8. There were 231 cases (89%) classified as cured, 2 cases (1%) died, 21 cases (8%) were classified as treatment failed and 6 cases (2%) were lost to follow-up.

The treatment outcomes of INH^R + RiF^R (MDR TB, group one) were 25 cases (46.3%) with favourable outcome and 29 cases (53.7%) with unfavourable outcome by WHO definitions. For INH^R (not MDR) patients, there was a favourable outcome for 231 cases (88.8%) and an unfavourable outcome for 29 cases (11.2%).

3.3.10 Effect of culture in addition to smear on unfavourable outcome detection rates

For patients with INH^R TB by MODS, enhanced surveillance for treatment failure was conducted, with sputum culture at months 5 and 8 in addition to sputum smear. Patients with a positive *M.tb* culture at month 5 or month 8, were additionally classified as failure cases.

The treatment outcomes in the two resistance groups confirmed by LJ DST (MDR group and INH^R group) were compared using WHO guidelines (smear only) and

based on culture at month 5 and 8 addition to smear to determine the additional number of failure cases detected by culture.

If culture was included in the criteria for evaluation of treatment outcomes, the unfavourable outcome detection rate for MDR cases increased from 53.7% (n=29/54) to 68.5% (n=37/54). Among INH^R (non MDR) cases the unfavourable outcome detection rate increased from 11.2% (n=29/260) to 19.2% (n=50/260). The details are presented in the Table 3-16 and Table 3-17. Eight additional unfavourable outcomes were detected among MDR TB cases by using culture in addition to smear. This difference was not significant (P=0.114).

260 cases with INH^R (not MDR) by LJ DST were evaluated. The results are presented in Table 3-17. Twenty one additional unfavourable outcomes were detected among INH^R (non MDR) cases using culture in addition to smear. This difference was statistically significant (p = 0.01).

Outcome		Based on smear only n (%)	Based on smear and culture n (%)
Favourable			
	Cured	25 (46.3)	17 (31.5)
	Treatment completed	0	0
	Died	0	0
	Total	25 (46.3)	17 (31.5)
Unfavourable			
	Treatment failed	27 (50.0)	35 (64.8)
	Lost to follow-up	1 (1.9)	1 (1.9)
	Not evaluated	1 (1.9)	1 (1.9)
	Total	29 (53.7)	37 (68.5)
Total		**54 cases (100%)**	**54 cases (100%)**

Table 3-16: Treatment outcomes for MDR TB cases using culture in addition to smear

Outcome category	Outcome	Based on smear only cases n (%)	Based on smear and culture cases n (%)
Favourable			
	Cured	231 (88.8)	210 (80.8)
	Treatment completed	0	0
	Total	231 (88.8)	210 (80.8)
Unfavourable			
	Died	2 (0.8)	2 (0.8)
	Treatment failed	21 (8.1)	42 (16.2)
	Lost to follow-up	6 (2.3)	6 (2.3)
	Not evaluated	0	0
	Total	29 (11.2)	50 (19.2)
Total		**260 cases (100%)**	**260 cases (100%)**

Table 3-17: Treatment results based on smear only and smear+culture in INH^Rnot MDR (group 2) cases

3.3.11 Comparison of treatment outcomes between patients with MDR TB and INH^R (non-MDR) TB

Treatment outcomes based on smear and culture of MDR cases (group 1) and INH^R (group 2) were compared with each other. Patients with MDR TB were more likely to

have unfavourable outcome compared to patients with INH^R (not MDR) (OR= 9.41, 95%CI: 4.84-18.29, p< 0.001) (Table 3-18 and Figure 3-12).

	Unfavourable outcome [cases, n (%)]	**Favourable outcome [cases, n (%)]**	**Total**	**OR [95%CI],p value***
INH^R n=247	46 (18.7) %)	201(81.4) %)	247 (100%)	BASELINE
MDR n=52	35 (67.3%)	17 (32.7%)	52 (100%)	9.41 [4.84-18.29], P < 0.001

*excluding patients receiving individualized regimens.

Table 3-18: Treatment outcomes in MDR group (group 1) and INH^R group (group 2)

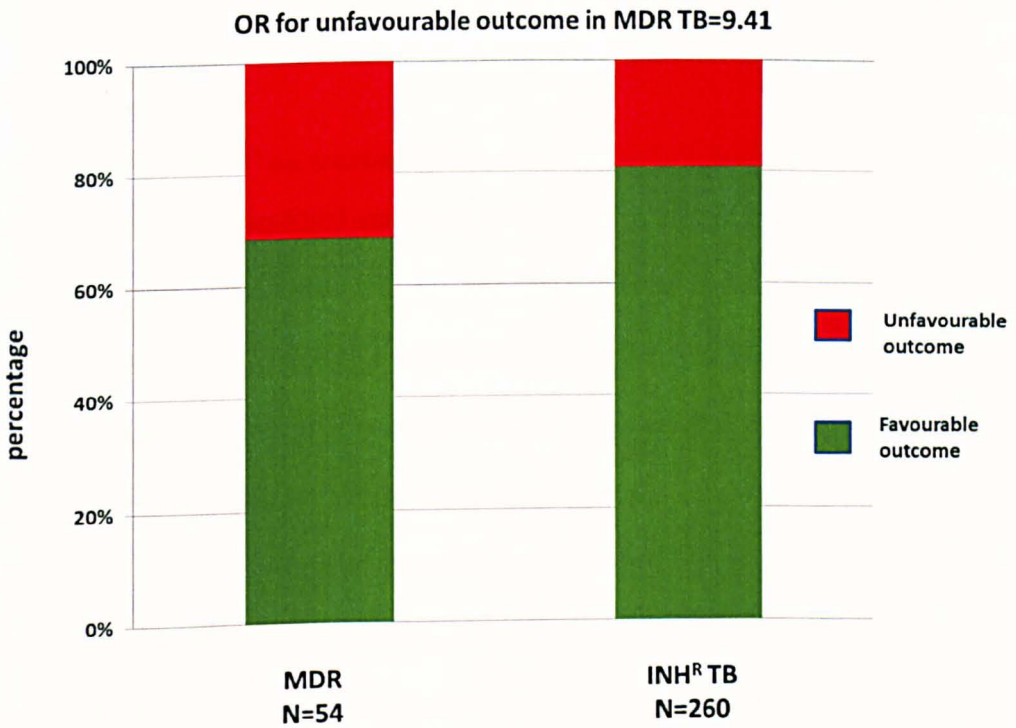


Figure 3-12 : Unfavourable treatment outcome among patients infected with MDR and INH^R *M.tb* strains

3.4 Discussion

3.4.1 Impact of INH^R on treatment outcomes

Overall, the WHO classified treatment outcomes for all patients in this study were consistent with those reported by the NTP, with 10.8% patients having an unfavourable outcome by WHO criteria, exceeding the WHO target of 85% treatment success. However, intensified surveillance for treatment failure/relapse among INH^R (non-MDR) cases detected almost twice as many unfavourable outcomes (11.2% vs. 19.2%). As intensified surveliance for treatment failure was not conducted among all patients due to resource limitations, it is unclear if this additional failure/relapse is limited to INH^R cases; it seems likely that additional failure/relapse cases would be detected among patients with drug susceptible isolates but the number of additional cases would be few.

The number of additional failure/relapse cases detected among INH^R (non-MDR) cases is cause for grave concern in the Vietnamese National TB control strategy. It is clear from this data that under the WHO recommended smear-only monitoring for treatment outcome, a substantial number of INH^R cases will be declared cured at treatment completion, only to relapse at a later date due to failure to eradicate the infection. This may be a significant contributing factor to the lack of observed decline in TB notification rates in Vietnam, despite a comprehensive NTP and control strategy for over 15 years [37]. Several other explanations have been proposed for the lack of impact such as the emergence of the HIV epidemic [38], rapid urbanization [334] and the role of *M.tb* Beijing genotype [335]. International TB guidelines

recommend the use of microscopic examination (direct smear) of at least two sputum specimens for acid fast bacilli to diagnose TB and follow-up to evaluate the treatment outcomes at month 5 and month 8 in high burden countries with financial constrains [164]. Vietnam National TB guidelines follow this recommendation, using three sputum smear according to old guidelines. A patient whose smear is positive at month 5 or later, month 8 is classified as treatment failure. However, the use of smear for evaluation of TB treatment is clearly insufficient to detect all treatment failures.

It would not be possible to provide culture follow-up to every patient within the NTP with current resources and case-burden, even within HCMC where the reference laboratory for southern Vietnam is located. There is limited capacity for *M.tb* culture and culture of approximately 16,000 new TB patients within the city at treatment completion would be an unrealistic goal. Alternative methods for treatment monitoring are urgently required; the role of GeneXpert MTB/RIF testing in treatment monitoring has yet to be established and is not currently recommended. While PCR-based tests detect both viable and dead bacilli and are therefore considered to be of limited use in treatment monitoring at month 2/3, the use of Genexpert at month 8 may have value and should be evaluated. The GeneXpert MTB/RIF test may have an advantage over other NAAT in this regard due to the capture of intact bacilli from the sample within the cartridge, prior to DNA extraction and amplification, which will reduce positive follow-up results due to extracellular DNA within the sputum. A two-pronged strategy in which patients with INH^R TB are identified at diagnosis for alternative treatment and more intensive outcome monitoring may be more cost-effective. Currently available methods for rapid identification of INH^R TB are

phenotypic methods such as the MODS assay used here, or genotypic approaches such as the Line Probe Assay [91, 95]. The major disadvatages of MODS are labour requirements and biosafety, while HAIN test has significant costs, quality control (QC) and training requirements. A novel Xpert cartridge which detects INH and RiF resistance may become available in the future but current strategies in high burden settings focus on TB case detection with Xpert and therefore target Xpert testing to HIV and MDR suspects only, the resource implementations of screening all TB suspects with Xpert would be prohibitive [336]. Modelling of cost-effectiveness of alternative approaches should provide insights into the optimal strategy to use and the long term cost: benefit ratio.

WHO has recommended a 4HRE treatment continuation phase 'in areas of high INH resistance' and this is currently being piloted in the Vietnamese NTP [129]. It is unclear if this regimen will have significantly improved outcomes in those patients with INH^R TB as the regimen is based on expert opinion rather than trial evidence. Overall the favourable outcome rates were not different between the 2 regimens (2SRHZ/6HE and 2RHZE/6HE).

Outcomes for patients on MDR TB were extrememly poor. For treatment outcomes based on definition of WHO and also Vietnam NTP, the favourable treatment outcomes of patients with MDR TB by LJ DST was just 46.3% (25/54 cases). If culture were included for evaluation of treatment outcomes, the cured rate decreased from 46.3% to just 31.5% in MDR patients at 8 months and it is likely that many of these would go on to relapse if not yet enrolled into appropriate treatment. Patients

with MDR TB were more likely to have unfavourable outcome compared to INH^R patients (OR= 9.41, 95%CI: 4.84-18.29, p<0.001).

Patients in this study with MDR TB had additional resistance and therefore received only one or two drugs to which the isolate was susceptible when given the standardised regimens 2SRHZ/6HE or 2RHZE/6HE. This clearly generates further resistance to the remaining first-line drugs to which the isolate is susceptible. During the study, patients diagnosed with MDR by MODS were not eligible for the MDR treatment programme in HCMC and were referred for HAIN testing if smear positive at month 5, consistent with NTP guidelines. At initiation of the programmatic management of MDR TB in HCMC, a limited number of treatment courses were available and patients were often not able to receive immediate registration for MDR treatment. Successful outcomes from the initial PMDRTB cohorts has led to an expansion of the treatment programme and improved access for patients, including eligibility based upon a positive GeneXpert test for RiF resistance at any treatment stage. This is a significant advance in the management of MDR TB within HCMC and should lead to significantly improved outcomes for individual patients in addition to reduced MDR TB transmission over time, but other research studies will be needed to establish the true impact of the PMDRTB on the DR TB epidemic in Vietnam.

3.4.2 Demographics of TB patients

The majority of patients (90%) were under 60 years of age and male (73.7%). This is consistent with data from other settings where TB is more common in men than women, usually with a ratio of between two and three to one [337]. The reasons for this are unknown but have been hypothesized as due to higher smoking rates or pre-

existing lung damage in men, different patterns of social mixing and immune response variation [337]. For occupations, 69.7% of TB patients are manual labourers . Low socioeconomic status has been repeatedly associated with risk of TB disease, probably due to factors such as malnutrition, over-crowding, co-morbidities and access to healthcare [338] but in this study was probably over-estimated due to recruitment of patients only through the public sector. In this study, gender and age were not an associated with treatment outcome although in other studies from Asia, association has been found between male gender [339] or young age [340] and poor treatment outcome.

No association was seen in this study between gender and age and risk of INH^R TB but MDR TB was significantly associated with young age, as in other studies from Vietnam. As all patients in this study were new smear positive TB patients, not having received any previous TB treatment, this suggests there is active community transmission of MDR TB among young adults and contact tracing of MDR TB patients via social networks should be intensified.

3.4.3 MODS for detection of INH^R TB

Based on MODS, we detected 392 cases (18.6%) with INH resistant TB (+/- RiF resistance). However, from the beginning of September 2010, we decreased the concentration of INH from 0.4 to 0.1 µg/ml, the number of INH resistant TB increased from 17.4% to 21.2%, but the percentage of these confirmed by LJ DST as the gold standard decreased from 89.7% to 81.75%. Due to resource limitations we were not able to test all cultures by LJ DST to confirm the accuracy of MODS for INH^R detection or to determine how many INH^R isolates were undiagnosed, before

and after the change in critical concentration. The sensitivity will be increased with the use of the 0.1 µg/ml isoniazid cutoff but conversely the specificity will be compromised compared to 0.4 µg/ml cutoff [322]. The median time for detection of *M.tb* by MODS was 10 days (mean: 10 days, range: 4-60 days). This is substantially faster than the 4-6 weeks required for conventional culture and DST. Moreover, this test is useful to diagnose HIV-infected patients with TB. According to a systematic review and meta-analysis, the MODS assay had wide variability with isoniazid for sensitivity (82-100%) and specificity (78-100%) [341]. Another limitation of MODS is the inability to differentiate *M.tb* and NTM which has been addressed by the addition of P-nitrobenzoic acid (PNB) to a well [85], biohazard risk, labour requirements for plate-reading, and a lack of established External quality assessment (EQA) procedures. However, WHO has recommended the MODS assay as an interim solution, especially in resource constrained settings [91]. The investment in training, infrastructure, EQA policy and modification of national algorithms will need to be weighed carefully in considering implementation of any new diagnostic as an 'interim' measure.

The MDR rate detected was 2.6% (n=54/2090). There were no cases of RiF monoresistance detected in this study; RiF resistance always occured in strains that were also resistant to other drugs such as INH, SM, EMB and PZA. This is consistent with global data showing rifampin resistance can be considered as a marker for MDR TB and confirms that use of the GeneXpert MTB/RIF test for diagnosis of MDR TB is appropriate in Vietnam [342]. GeneXpert MTB/RIF and Line Probe assays which are rapid, reliable and easy to interpret, can both be effectively applied for early

detection of MDR TB because these molecular techniques correlate very highly with solid or liquid medium culture and DST which is a gold standard for TB diagnosis [343]. However, molecular testing also has limitations, principally failure to detect all phenotypically resistant cases, and the need for traditional culture and DST is not removed [344]. Further, line probe assays can only be used effectively in smear positive cases. WHO issued a policy statement recommending LPA for rapid screening of patients suspected with MDR TB in 2008 [345]. In 2010, WHO also approved the Xpert MTB/RIF test [96]. Technical and operational practical considerations for using Xpert MTB/RIF have also been presented and published by WHO in 2011 [336] . The global community has since undertaken huge scale up implementation of Xpert MTB/RIF testing in high-burden settings and data on the true impact of this implementation on TB epidemics is likely to emerge first from South Africa, which has undertaken country-wide implementation [346, 347].

3.4.4 Treatment options for INH^R TB

For INH^R TB which is susceptible to rifampicin, the unfavourable outcome rate was 19.2%. Patients with INH^R received the standard regimen according to NTP guidelines; 2SRHZ/6HE or 2RHZE/6HE. These patients effectively received 2SRZ/6E or 2RZE/6E, although there is evidence from MDR series that taking INH retains some benefit in INH^R cases [222].

Resistance to isoniazid is likely to increase as countries implement the WHO recommended strategy of 'the 3 I's', including isoniazid preventative therapy (IPT) provision in HIV-infected individuals on a large scale [348]. Theoretical studies suggest there may be an impact of large-scale IPT on INH-resistance rates in

M.tuberculosis [349] but this has not yet been observed in practice probably due to the time required for any impact to become apparent [350]. In addition, studies have consistently demonstrated an increased risk of drug resistance amplification, and MDR development, in patients with undiagnosed INH-resistant TB at commencement of standardized drug regimens [330]. A recent WHO review of existing data concluded "Patients with pretreatment isoniazid resistance (but susceptibility to rifampicin and streptomycin) were 22 times more likely to acquire drug resistance than patients who started treatment with drug-susceptible disease" [265, 330].

Determining and applying an optimized regimen for INH-resistant, RiF sensitive TB will 'turn off the tap' for MDR TB and substantially reduce the emergence of new MDR strains [351]. The WHO currently recommends the addition of ethambutol to the continuation phase programmatically 'in regions of high prevalence of INH resistance', but the threshold for 'high' is undefined: "In populations with known or suspected high levels of isoniazid resistance, new TB patients may receive HRE as therapy in the continuation phase as an acceptable alternative to HR (weak/insufficient evidence, expert opinion)" [129]. Vietnam is a country with high INH^R rate [352] so since May, 2013 the regimen of 2RHZE/4RHE for new smear positive pulmonary TB has been implemented in HCM City. The effectiveness of this regimen should be evaluated and the data will help to determine if implementation of this regimen is of benefit.

For patients with confirmed INH resistance a regimen of 6-9 months RZE with the addition of a FQ to strengthen the regimen in 'extensive' disease is recommended [234]. The British National Institute for Clinical Excellence (NICE) guidelines

recommend a 7 month continuation phase of RE [353] and the American Thoracic Society guidelines suggest a fluoroquinolone (FQ) is added in cases of "extensive disease" [354]. The programmatic addition of ethambutol to the regimen for all patients must be considered carefully against the risk of ocular toxicity with the potential for permanent blindness in patients who are receiving ethambutol unnecessarily. Further, it is not actually known if the addition of ethambutol has any impact on outcome or the risk of drug resistance amplification due to the lack of evidence from randomised controlled trials. The evidence base for all recommendations is acknowledged to be extremely weak and no randomized controlled trials have been conducted for the treatment of INH-resistant, RiF sensitive TB.

3.4.5 Study limitations

There was considerable recruitment bias in this study due to two principle factors. Firstly, in Ho Chi Minh City, the percentage of TB patients managed within the private sector may be as high as 30% to 40% and is increasing with economic development of the country, which achieved middle income status in 2010 [355]. These patients were not captured within the study. Furthermore, many patients were ineligible or declined to participate in the research. Socio-economic characteristics are likely to differ substantially between patients accessing the private and public sectors and this is likely to influence outcomes through various factors. Although patients in the private sector are likely to have better health care access, nutrition and opportunity to convalesce they may also receive inadequate treatment within the private sector.

Adherence to treatment was recorded during the study in the patient CRF but was not incorporated in the analysis because treatment adherence was recorded as 100% for all patients which is unlikely to be accurate, therefore we were probably unable to capture adherence. Alternative methods such as fingerprint monitoring would have more acurrately captured adherence and enabled us to separate the impact of primary drug resistance and poor adherence on treatment outcomes.

MODS was used for screening INH^R and a change in critical concentration was made during the study due to new recommendations. LJ DST showed that the specificity of MODS for detection of INH^R TB was relatively low: 89.7% for 0.4μg/ml cut-off and 81.75% for 0.1μg/ml. We were unable to determine the sensitivity of MODS for INH^R detection due to resource limitations which prevented us conducting LJ DST on all patients in the screening study, therefore some cases of INH^R TB may have been mis-classified as susceptible, particularly with a 0.4μg/ml cut-off and the concentration change will have resulted in uneven distribution of mis-classification during the study.

It was also not possible to conduct culture monitoring for treatment failure in all screening patients which would have enable comparison of enhanced surveillance for treatment outcomes between all groups. The high unfavourable outcome rate in INH^R TB detected by culture at month 8 should be compared with drug susceptible TB to determine the true impact of INH^R on treatment outcome. Further surveillance of patients up to 2 years post-treatment will also allow determination of relapse rates.

Finally, patients who died during TB treatment, were classified as unfavourable outcome (all-cause mortality). This would not be totally accurate because some

patients may have improved clinical situations and they may die from another causes such as accident. The causes of death in this study were not investigated systematically; at least one patient died due to knife fight rather than tuberculosis.

3.4.6 Conclusion

In conculsion, an unfavourable outcome rate of 19% by treatment completion is unacceptably high, and is likely to be further augmented by relapse over the subsequent 1-2 years. It is clear there is an urgent need to evaluate screening strategies for INH^R TB and to determine the optimal programmatic treatment regimen through randomized controlled trials.

Chapter 4

Bacterial factors associated with treatment failure for Isoniazid Resistant Tuberculosis

4.1 Aims

1) Determine the association between lineage of *M.tb* and drug resistance.
2) Determine the association between mutations conferring INH resistance, MIC level, bacterial lineage and outcome.
3) Investigate the relationship between MIC for isoniazid and unfavourable outcome in cases of INH-resistant TB susceptible to rifampicin.
4) Determine the impact of resistance to additional drugs on outcome.
5) Compare treatment outcomes on two standardized treatment regimens for patients infected with different patterns of INH polyresistance.

4.2 Introduction

While the majority of patients with INH resistant TB are successfully cured on standard regimens, the failure/relapse rate is known to be higher than for drug susceptible strains. The purpose of this chapter was to investigate three bacterial factors as risk-factors for treatment failure and relapse, namely bacterial lineage, INH MIC, and mutation responsible for INH resistance.

The *M.tb* Beijing genotype, which was first described in 1995 from the Beijing region of China is one of the most prevalent clades worldwide [124, 356]. Beijing genotype

represents the predominant genotype in some regions, particularly Asia, and has been estimated to account for 13% of *M.tb* strains circulating globally [124]. The prevalence of this strain is high in Asia, intermediate in the USA and Cuba and low in parts of Africa, Latin America, Western Europe, Eastern Europe and in the Middle East. The Beijing genotype has been associated with drug resistance and MDR in some studies but not in others [357, 358]. This variation may be due to different and undistinguished sublineages of the Beijing lineage but further studies are required to determine the underlying reasons for these observed differences. In 2004, Beijing genotype strains were defined as those which hybridized by spoligotyping to at least three of the spacer 35-43 in the genomic direct-repeat region, and lack spacers 1-34 [359]. Further sub-division of the clade has been defined by several techniques including second-generation spoligotyping, IS*6110* typing and Mycobacterial Interspersed repetitive units (MIRU) also known as variable number tandem repeat (VNTR). In early studies of the phylogeny the Beijing clade was divided into two principal groups named typical and atypical Beijing strains [359]. Based on large-sequence polymorphisms, the Beijing lineage has been divided into four subgroups on the basis of large phylogenetic deletions known as regional difference (RD): RD105, RD181, RD150 and RD142 [360]. Beijing genotype strains have been considered to be an emerging in some regions based on evidence such as the increase in the Beijing genotype strains over more than 3 years in some Western European countries, strong correlation between this strain and young age, and the increased presence in archived samples conducted in South Africa [358, 361]. The emergence of Beijing genotype strains has been hypothesized to be due to an 'escape mutant' of BCG vaccination but

there is limited evidence to support this theory [358, 362, 363]. An alternative theory is a fitness advantage under selective drug pressure. However, the comparative virulence of this strain has been also investigated in terms of transmission, progression from latent to active TB, acquisition of drug resistance and chronicity of the TB disease [364]. It is possible that the Beijing lineage represents an adaptation to urbanization of human populations into high density populations which favours a higher transmission over latency. There is a significant association between this strain and young age in some regions which is a surrogate marker of active transmission in communities [365]. In a study from Vietnam, during 2003-2006, all patients with new sputum smear-positive pulmonary tuberculosis in 3 rural districts in Tien Giang Province situated in Mekong River Delta in Southern Vietnam were genotyped. Beijing genotype was more frequent in younger patients (15-24 years of age, compared to other patients (53% vs. 31%, $p<0.001$) and associated with MDR [335]. The increase of Case Notification Rates was larger with the Beijing genotype than other genotypes but this difference was not statistically significant [335].

The association between Beijing genotype and specific resistance-conferring mutations has also been investigated. An association between Beijing genotype and *katG*315 mutations for isoniazid resistance has been demonstrated but no clear association is evident between this strain and any specific mutation site in the *rpoB* gene [366, 367]. This may reflect the fact that the common clinical *rpoB* mutations do not vary substantially in the level of resistance they confer, whereas those for INH do. Research from Vietnam has shown that *katG*315 mutations, were also associated with unfavorable treatment outcomes compared to *inhA* mutations but this study found no

association between *katG* mutation and Beijing genotype [368]. A study from Russia found that Beijing strains were more likely to have mutations in the *embB*306 gene. High level fluoroquinolone and streptomycin resistance conferring genes in *gyrA* and *rpsL* were also found to be associated with Beijing genotype among resistant isolates in Vietnam [369, 370]. The underlying factors responsible for increased drug resistance in Beijing strains have been hypothesized to be a higher mutation frequency, the specific cell wall structures leading to intracellular inadequate TB drug concentration, efflux mechanisms or compensatory mutations which allow lower fitness cost of specific resistance mutations [358].

M.tb Beijing genotype strains may have an ability to subvert the immune response and alter clinical disease progression [371]. Patients infected with the Beijing genotype strains were more likely to progress to disease in one study [119]. Many clinical features have been compared between patients infected with the Beijing lineage strains and those infected with other lineages but the results have been conflicting. In a study from Vietnam, patients with HIV and TBM caused by the Beijing genotype strains had a shorter duration of illness and a lower cerebrospinal fluid leucocyte count compared to other strains [372].

In summary, the Beijing lineage of *M.tb* appears to be increasing in global prevalence and to be associated with drug resistance. More research is required to understand the molecular epidemiology, potential variation in vaccine efficacy and mechanisms of drug resistance [358].

It is known that INH resistant strains of *M.tb* have different MICs, in particular the two commonest INH-resistance conferring mutations *katG*315 and *inhA* -15C→T

mutations have high (≥5ug/ml), and low MIC's (≤1ug/ml), respectively [178]. The clinical relevance of this is not known but serum isoniazid concentrations fall within this range, with 3 μg/ml at 2 hours after dosing being generally considered the lower limit of normal. It is therefore probable that patients with inhA -15C→T isolates have a better chance of successful treatment with standard regimens. The *katG*315 mutation is also associated with the development of MDR and transmission, an effect not seen with *inhA* -15C→T mutation [178]. In addition, isolates with a *katG* mutation are more likely to develop ethambutol resistance [373] which would further decrease the chances of successful treatment in 8 month INH/ethambutol continuation phase regimens.

While many patients are successfully treated on the standard 8-month regimen, primary isoniazid resistance is strongly associated with failure or relapse. It is likely that isolates with a high MIC to isoniazid carry a higher risk of failure than those with a low MIC. It is possible that molecular screening of isolates for *katG*315 mutations would help to identify those at risk of treatment failure and acquiring MDR on treatment with standard regimens.

Greater understanding of risk factors for treatment failure/relapse in patients with INH^R TB will potentially help us to design screening which will target interventions to those at high risk of failure and avoid unnecessary increased drug administration to all patients.

4.3 Results

4.3.1 Association of bacterial lineage with demographic characteristics and drug resistance

Overall, among 2,090 isolates from patients included in the screening study LSP genotyping data was available for 1,780 (85.2%) cases. Beijing genotype was identified by LSP typing in 1,063 (59.7%) cases, Indo-Oceanic in 421 (23.6%), Euro-American in 192 (10.8%) and 104 (5.8%) isolates had indeterminate genotype.

LSP genotyping results were available for 241 patients with INH resistance confirmed by LJ DST. The results showed 162 cases (67.2%) were infected with the Beijing lineage, 33 cases (13.7%) with Indo Oceanic lineage, 35 cases (14.5%) with Euro American lineage and for 11 cases (4.6%) the lineage was undetermined (mixed infection or failed PCR) (Figure 4-1).

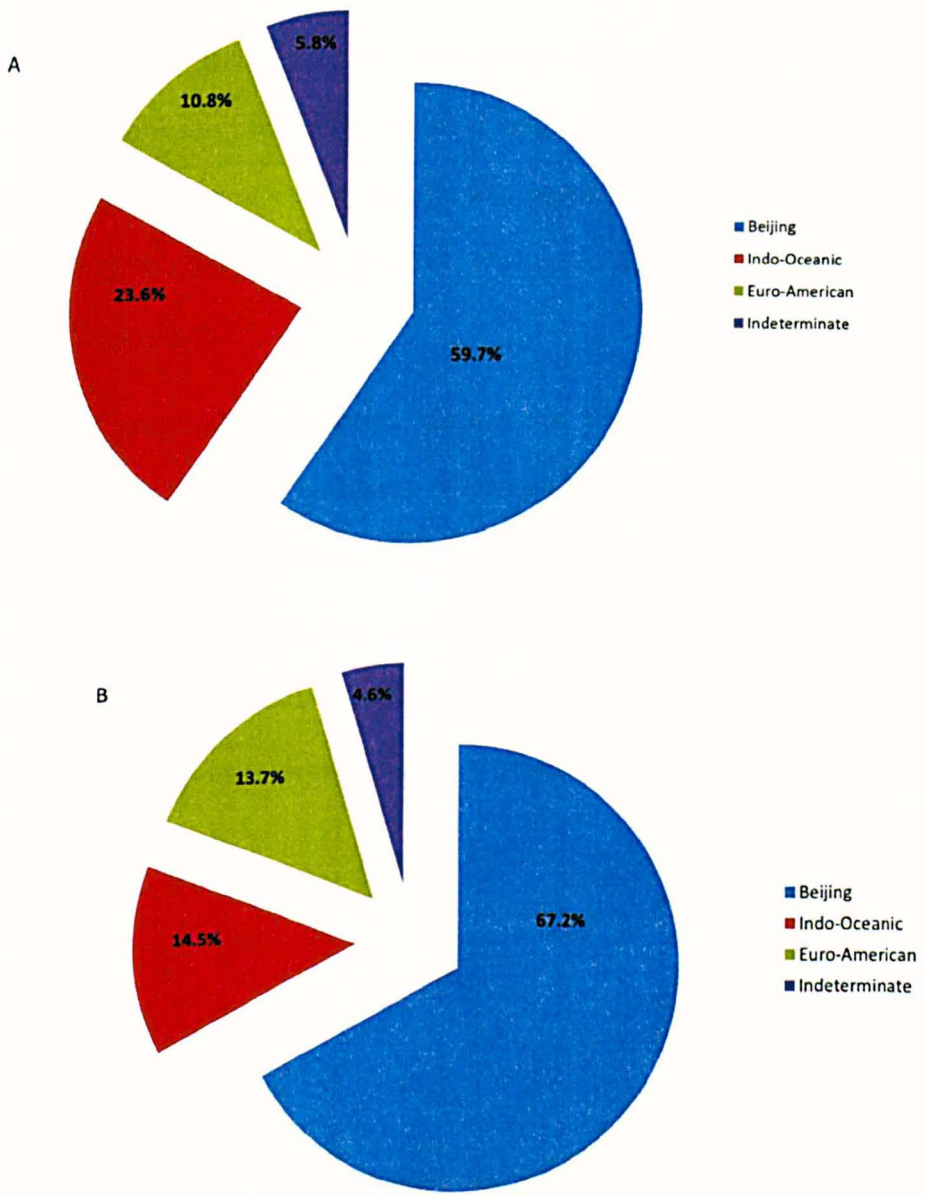


Figure 4-1: Distribution of *M.tb* lineages identifed in (A) all patients and (B) patients infected with INH^R (non MDR) *M.tb* confirmed by LJ DST

Overall, when demographic factors associated with bacterial lineage were analysed Beijing lineage was independently associated with young age (<35 years of age) (OR=1.29 [95% CI 1.06-1.57], P=0.011) and male sex (OR =1.29 [95% CI 1.03-1.62], P=0.024). Indo-Oceanic lineage was significantly associated with older age (OR=0.71 [95% CI 0.57-0.90], P=0.04) . No lineage showed an association with Kinh ethnicity (Table 4-1).

Analysis of drug resistance patterns showed that Beijing genotype was significantly associated with both INH resistance (OR=1.63 [95% CI 1.26-2.12], P=<0.001) and MDR (OR=4.01 [95% CI 1.88-8.55], P=<0.001). Conversely, Indo-Oceanic strains were significantly less likely to be either INH resistant (OR=0.36 [95% CI 0.25-0.52], P=<0.001) or MDR (OR=0.12 [95% CI 0.29-0.49], P=0.003). Euro-American strains showed no association with drug resistance (Table 4-2).

Lineage	Factor	Crude OR			Adjusted OR		
		OR	95% CI	P-value	OR*	95% CI	P-value
Beijing							
	Young Age (<35 yrs)	1.33	1.09-1.61	0.004	1.29	1.06-1.57	0.011
	Sex	1.35	1.08-1.68	0.008	1.29	1.03-1.62	0.024
	Kinh ethnicity	0.89	0.60-1.32	0.558	0.89	0.59-1.33	0.565
Indo-Oceanic							
	Young age (<35 yrs)	0.69	0.55-0.87	<0.001	0.71	0.57-0.90	0.04
	Male sex	0.74	0.57-0.97	0.027	0.78	0.60-1.02	0.659
	Kinh ethnicity	0.90	0.58-1.41	0.647	0.90	0.58-1.41	0.659
Euro-American							
	Young Age (<35 yrs)	0.84	0.62-1.14	0.271	0.85	0.62-1.16	0.297
	Male sex	0.91	0.64-1.29	0.603	0.94	0.66-1.34	0.736
	Kinh ethnicity	0.98	0.53-1.81	0.938	0.97	0.52-1.81	0.938

Comparison for each lineage against all other strains

***Adjusted for age, gender, ethnicity**

Table 4-1: Association between demographic factors and lineage of infecting *M.tb* strain

Lineage	Drug resistance pattern	Crude OR	95% CI	P-value	Adjusted OR*	95% CI	P-value
Beijing							
	INH resistant	1.65	1.27-2.14	<0.001	1.63	1.26-2.12	**<0.001**
	MDR	4.11	1.93-8.76	<0.001	4.01	1.88-8.55	**<0.001**
Indo-Oceanic							
	INH resistant	0.36	0.025-0.51	<0.001	0.36	0.25-0.52	**<0.001**
	MDR	0.12	0.028-0.48	0.03	0.12	0.29-0.49	**0.003**
Euro-American							
	INH resistant	1.30	0.90-1.88	0.161	1.32	0.75-1.29	0.108
	MDR	0.30	0.74-1.26	0.100	1.32	0.91-1.91	0.141

*Adjusted for young age (<35 years of age) and sex

Comparison for each lineage against all other strains

Table 4-2: Association between drug resistance and bacterial lineage.

4.3.2 Impact of INH MIC on outcome for patients with INH resistant (not MDR) TB

Six of 260 isolates (2.3%) were lost or culture failed for MIC. The remaining 254 isolates with INH^R diagnosed by LJ DST were tested for MIC of INH using the critical concentrations of INH at 0.2 μg/ml, 1 μg/ml, 2 μg/ml, 4 μg/ml, 8 μg/ml and 16 μg/ml. The results showed 127 isolates (50%) of INH^R strains with MIC 0.2 μg/ml ≤ MIC < 2 μg/ml and 127 cases (50%) with INH^R strains with MIC ≥ 2μg/ml. The detailed results are shown in Table 4-3.

Concentration (μg/ml)	≥ 0.2	≥1	≥2	≥4	≥8	≥16	total
n (%)	32 (12.6%)	95 (37.4%)	113 (44.5%)	9 (3.5%)	2 (0.8%)	3 (1.2%)	254 (100%)

Table 4-3: The distribution of INH MIC among 254 INH^r cases

Treatment outomes (smear+culture) were evaluated by INH MIC level for 254 INH^R cases (non MDR) diagnosed by LJ DST. The results are shown in the Table 4-4.

Concentration (μg/ml) /outcomes	Outcomes						Total
	Cured n (%)	Treatment completed n (%)	Died n (%)	Lost to follow-up n (%)	Treatment failed n (%)	Not evaluated n (%)	Total n (%)
≥0.2	25 (12.0%)	0	0	0	7 (18.4%)	0	32 (12.6%)
≥1	77 (37.0%)	0	1 (50%)	2 (33%)	15 (39.5%)	0	95 (37.4%)
≥2	93 (44.7%)	0	1 (50%)	4 (67%)	15 (39.4%)	0	113 (44.5%)
≥4	8 (3.8%)	0	0	0	1 (2.6%)	0	9 (3.5%)
≥8	2 (9.6%)	0	0	0	0	0	2 (0.8%)
≥16	3 (1.4%)	0	0	0	0	0	3 (1.2%)
Total	208 (100%)	0	2 (100%)	6 (100%)	38 (100%)	0	254 (100%)

Table 4-4: Treatment outcomes by INH MIC level

MIC to INH was categorized into high (≥ 1 μg/ml) and low (<1μg/ml) for further analysis. Treatment outcomes were categorized as favourable (cured and treatment completed) or unfavourable (death, lost to follow-up, treatment failed and not evaluated).

There was no association between unfavourable outcome and high MIC (OR=0.83 [95%CI 0.38-2.06], P=0.69. Further, there was no association between high MIC and bacterial lineage (ANOVA, P=0.289) (Table 4-5).

MIC / outcome	**Unfavourable outcome n (%)**	**Favourable outcome n (%)**	**Total n (%)**	**OR, [95%CI], p-value***
High MIC (≥ 1 μg/ml)	39 (17.6)	183 (82.4)	222 (100)	BASELINE
Low MIC (<1 μg/ml)	7 (21.9)	25 (78.1)	32 (100)	OR=0.83 [95% CI 0.38-2.06] P=0.69.
Total	45 (17.7)	209 (82.3)	254 (100)	

Table 4-5: Association between MIC category and and unfavourable treatment outcome

4.3.3 Mutations identified by MAS_INH PCR among INH^R isolates

MAS-INH PCR demonstrated that 197 cases (77.3 %) were infected with isolates with a *katG* mutation, 21 cases (8.2 %) had *inhA* promoter mutation and 37 cases (14.5 %) showed WT isolates (Figure 4-2).

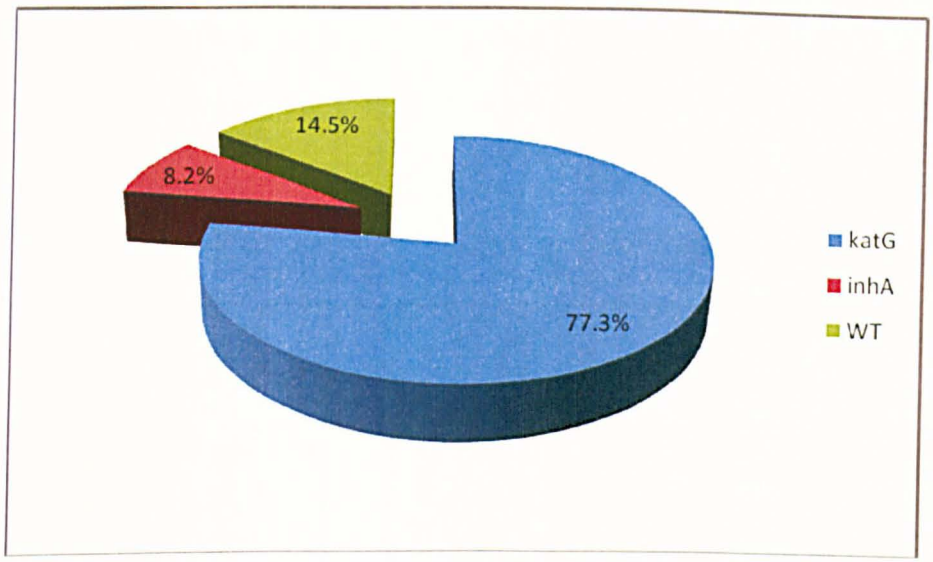


Figure 4-2 : Distribution of mutations identified by MAS PCR in patients with INH^R (not MDR) by LJ culture

4.3.4 Association between INH-resistance mutation and MIC for isoniazid

MIC and mutation data was available on 299 INH resistant isolates, including MDR strains. MIC was categorized into high (≥1µg/ml) and low MIC (<1µg/ml). *KatG* mutated isolates were significantly more likely to have high MIC to INH (OR=11.73 [95% CI 5.25-26.2]. P=0.001) while *inhA* mutants and wild-type strains were significantly less likely to have a high MIC to INH (OR=0.05 [95%CI 0.19-13.0], P<0.001). Table 4-6 shows the association between mutations and MIC in 299 cases with INH^R diagnosed by LJ DST in this study.

	INH MIC	*KatG n (%)*	*inhA n(%)*	Wildtype n(%)	Total n(%)
Low					
	0.2	**10 (4.3)**	**14 (60.9)**	**10 (21.7)**	**34 (11.4)**
Total					
High					
	1	**100 (43.5)**	**2 (8.7)**	**19 (41.3)**	**121 (40.5)**
	2	**107 (46.5)**	**3 (13.0)**	**15 (34.9)**	**125 (41.8)**
	4	**12 (5.2)**	**0**	**0**	**12 (4.0)**
	8	**0**	**1 (4.3)**	**1 (2.2)**	**2 (0.7)**
	16	**1 (0.4)**	**3 (13.0)**	**1 (2.2)**	**5 (1.7)**
	Total	**230 (100)**	**23 (100)**	**46 (100)**	**299 (100)**

Table 4-6: Distribution of INH MIC among isolates with different mutations conferring INH resistance

4.3.5 Association between Bacterial lineage and INH resistance mutation

Beijing lineage was significantly associated with *katG* mutation among INH^R isolates, (OR=2.01 [95% CI 1.23-3.58], P=0.018), while Indo-Oceanic strains were significantly less likely to have a *katG* mutation (OR=0.34 [95%CI 0.16-0.70], P=0.003). No lineage showed an association with *inhA* promoter mutation. Indo - oceanic strains were significantly more likely to be wild type, carrying neither a *katG* nor an *inhA* promoter mutation (OR=3.79 [1.75-8.19], P=0.001)(Table 4-7).

Lineage N=295	**KatG**			**inhA**			**Wild type**		
	OR	95% CI	P-value	OR	95% CI	P-value	OR	95% CI	P-value
Beijing N=218									
	2.01	1.23-3.58	0.018	1.07	0.41-2.81	0.888	0.39	0.20-0.75	0.005
Indo-Oceanic N=36									
	0.34	0.16-0.70	0.003	1.01	0.29-3.57	0.989	3.79	1.75-8.19	0.001
Euro-American N=41									
	0.95	0.44-2.05	0.891	0.88	0.25-3.11	0.848	1.15	0.47-2.78	0.760

Table 4-7: Association between M.tb lineages and mutation conferring INH resistance among 295 INH^R isolates

4.3.6 Association between INH resistance mutation and unfavourable outcome

The relationship between INH^R mutation and treatment outcome was investigated for 255 cases with INH^R (non MDR) diagnosed by LJ DST(table 4.8). There was no association between *katG* (P=0.281), *inhA* (P=0.578) or wild-type (0.395) with unfavourable outcome. The results are presented in table 4.9

Treatment outcomes	**Cured n (%)**	**Treatment completed n (%)**	**Treatment failed n (%)**	**Lost to follow-up n (%)**	**Died n (%)**	**Total n (%)**
***katG* mutation**	164 (83.7)	0	27 (13.7)	6 (3.0)	2 (1.0)	197 (100)
***inhA* mutation**	17 (81.0)	0	4 (19.0)	0	0	21 (100)
WT	28 (75.7)	0	9 (24.3)		0	37 (100)
Total	207 (81.2)		40 (15.6)	6 (2.4)	2 (0.8)	255 (100)

Table 4-8: Treatment outcomes and mutation types of 255 cases with INH^R (non MDR)

INH resistance mutation	Favourable outcome n(%)	Unfavourable outcome n(%)	Total n(%)	OR, [95%CI] and p-value*
katG mutation	164 (82.2)	35 (17.8)	197(100)	**0.68 [0.34-1.37] 0.281**
inhA	17 (81.0)	4 (19.0)	21 (100)	**1.35 [0.47-3.88] 0.578**
Wild type	28 (75.7)	9 (24.3)	37 (100)	**1.43 [0.63-3.27] 0.395**
total	209 (80.8)	46 (19.2)	255 (100)	

Comparison for each mutation type with other pooled mutaion types.

Table 4-9: Association between treatment outcomes and INH^R mutations among 255 cases with INH^R (non MDR) TB

4.3.7 Association between unfavourable outcome and *M.tb* lineage

The final outcomes by *M.tb* lineage of the infecting strain were assessed in 229 patients with INH^R (non MDR) TB confirmed by LJ DST.

There was no significant association of any *M.tb* lineage with unfavourable outcome (table 4.10).

M.tb Lineage	Outcome		Crude OR*			Adjusted OR**		
	Favourable n (%)	Unfavourable n (%)	OR	95% CI	P-value	OR	95% CI	P-value
Beijing n=161	126 (78.3)	35 (21.7)	2.08	0.91-4.76	0.08	2.37	0.97-5.83	0.059
Indo-Oceanic n=29	26 (89.7)	3 (10.3)	0.46	1.33-1.60	0.223	0.43	0.12-1.57	0.201
Euro-American n=34	34(87.2)	5 (12.8)	0.59	0.22-1.61	0.300	0.55	0.19-1.59	0.273
Total n=229	186 (81.2)	43 (18.8)						

*OR for unfavourable outcome. Each lineage compared to others combined.

**adjusted for resistance to streptomycin, ethambutol and pyrazinamide and treatment regimen.

Patients with MDR TB and receiving individualized regimens excluded.

Table 4-10: Treatment outcomes in 229 INH^R cases (non MDR) by *M.tb* lineage of the infecting strain

4.3.8 Treatment outcomes by drug resistance profile among patients with INH resistant TB

LJ DST was performed for INH, RiF, SM, PZA and EMB for 340 samples with INH^R by MODS. The results showed 55 cases with MDR (INH and RiF resistance) and 260 cases with INH^R. Among these 260 cases, 52 cases were INH monoresistant, 184 cases were resistant to INH and SM and 24 cases were INH polyresistant with other DST profiles (not MDR). 260 cases with INH^R (not MDR) were followed up to evaluate their treatment outcomes. Discrepencies in treatment outcome between INH monoresistant cases and INH polyresistant cases (excluding MDR) were investigated (Table 4-11).

DST profile	Treatment outcomes					
	Cured	Treatment completed	Died	Lost to follow-up	Treatment failed	Total
INH^R	41 (78.8)	0	0	0	11 (21.2)	52 (100)
$SM^R + INH^R$	159 (85.9)	0	1 (0.5)	5 (2.7)	20 (10.9)	184 (100)
$INH^R + PZA^R$	0	0	0	0	2 (100)	2 (100)
$SM^R + INH^R + EMB^R$	1 (25.0)	0	0	1 (25.0)	2 (50.0)	4 (100)
$SM^R + INH^R + PZA^R$	13 (76.5)	0	0	0	4 (23.5)	17 (100)
$SM^R + INH^R + EMB^R + PZA^R$		0	1 (100)	0	0	1 (100)
Total	213 (81.9)	0	2 (0.8)	6 (2.3)	39 (15.0)	260 (100)

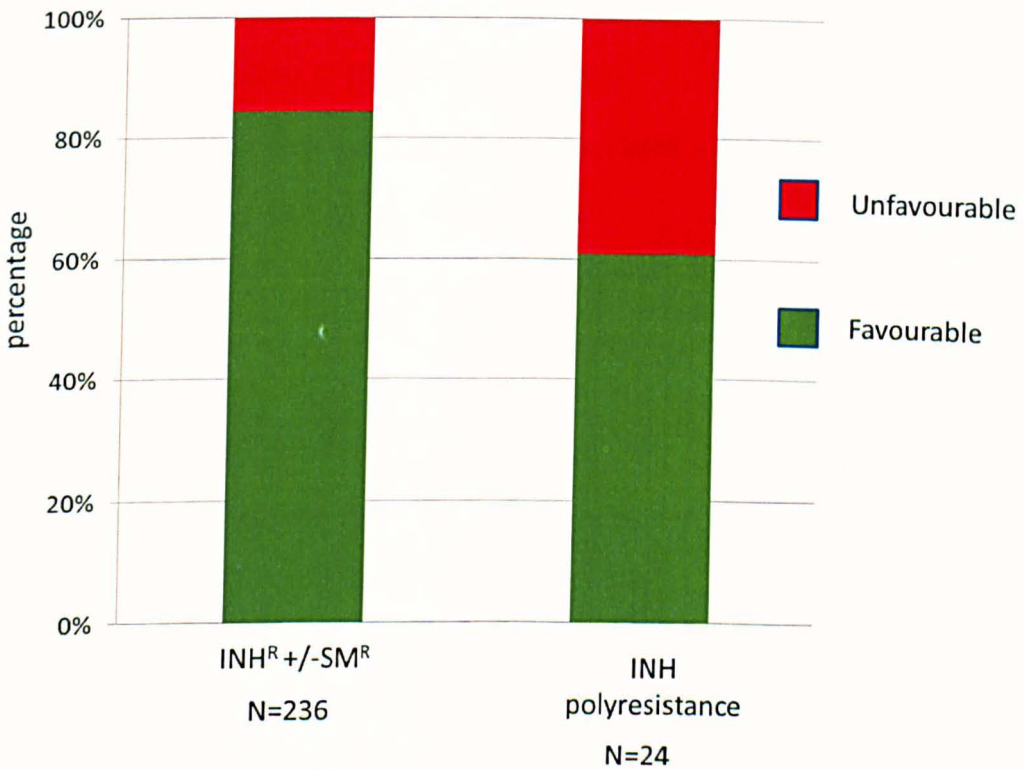
Table 4-11: Treatment outcome in patients with INH resistant TB (excluding MDR) by drug resistance profile

Treatment outomes (smear + culture) were compared for patients infected with INH^R +/- SM^R with those infected with strains with additional drug resistance (INH polyresistance). For those patients receiving the regimen of 2RHZE/6HE, SM^R should have no impact on outcome. Patients with INH polyresistance (not MDR) were more likely to have unfavourable outcome compared to patients with INH^R +/- SM^R (the regimens for these patients were 2SHRZ/6HE and 2RHZE/6HE) (OR=3.84, 95%CI: 1.59-9.30, p=0.003) (Table 4-12 and Figure 4-3).

	Unfavourable outcome n (%)	Favourable outcome n (%))	Total n(%)	OR, [95%CI], p-value*
INH polyresistance	10 (41.7%)	14 (58.3%)	24 (100%)	OR=3.84 [1.59-9.30] p=0.003
INH^R +/- SM^R	37 (15.7%)	199 (84.3%)	236	BASELINE
Total	46	214	260	

*logistic regression

Table 4-12: Association between treatment outcomes and DST profile for 260 patients with INH^R TB



***Polyresistance= additional resistance to ethambutol and/or pyrazinamide.**

Figure 4-3 : Association between INH resistance profiles and treatment outcomes in 260 INH^R cases

4.3.9 Multivaraite analysis of bacterial factors associated with unfavourable treatment outcome

Association of Beijing lineage, *katG* mutation, treatment with streptomycin (2SRHZ/6HE), streptomycin resistance and ethambutol resistance was analysed for association with unfavourable outcome (including culture) additionally adjusted for MODS concentration (table 4.13). Among 225 cases with all variables available, excluding those receiving individualized regimens, the adjusted OR for association of Beijing genotype with unfavourable treatment outcome (including culture) was 2.52 [95% CI 1.00-6.31], P=0.028. Streptomycin resistance was associated with favourable outcome (adjusted OR=0.37 [0.154-0.88], P=0.024).

Resistance to ethambutol was significantly associated with unfavourable outcome (adjusted OR=21.1 [2.00-222.50], P=0.011).

Variable N=225	Unfavourable outcomes n	Crude OR	95% CI	P-value	Adjusted OR	95% CI	P-value
Beijing lineage n=161	35	2.08	0.91-4.76	0.082	2.52	1.00-6.31	0.049
katG mutation n=173	31	0.81	0.38-1.76	0.600	0.59	0.22-1.56	0.283
Streptomycin treatment* N=55	15	1.96	0.95-4.01	0.067	2.09	0.87-5.06	0.101
Streptomycin resistance N=178	30	0.59	0.28-1.24	0.167	0.37	0.154 0.88	0.024
Ethambutol resistance n=4	3	13.88	1.41-136.85	0.024	21.1	2.00-222.50	0.011
MODS concentration N=82 (0.1µg/ml)	12	0.64	0.31-1.33	0.233	0.78	0.32 1.92	0.586

*2SRHZ/6HE

Excluding individuals receiving individualized regimens.

Table 4-13: The univariate (unadjusted) and multivariate (adjusted) OR, 95% CI and p values for the association between risk factors and treatment outcomes

4.3.10 Comparison of treatment outcomes of 2 regimens (2SRHZ/6HE and 2RHZE/6HE) in patients with INH and SM resistance confirmed by LJ culture and DST

There were 184 cases with INH^R and SM^R diagnosed by LJ culture. Overall, the favourable outcome rate in patients with SM+INH resistance was 86.4 % (159/184). From the end of 2009, the regimen 2SRHZ/6HE was replaced by the regimen 2RHZE/6HE at DTUs according to Vietnam NTP. We compared the treatment outcomes of patients treated with these two regimens among patients infected with strains resistant to both isoniazid and streptomycin but susceptible to other first line drugs (INH^R + and SM^R). The treatment outcomes by treatment regimen (2SRHZ/6HE and 2RHZE/6HE) are shown in the table 4.14.

Regimen	Treatment outcome				
	Cured n (%)	Treatment failed	Lost to follow-up	Died	Total
2SRHZ/6HE	33 (76.7)	7 (16.3)	3 (7)	0	43 (100)
2RHZE/6HE	118 (90.8)	12 (9.2)	0	0	130 (100)
other regimens	7 (66.7)	1 (8.3)	2 (16.7)	1 (8.3)	11 (100)
Total	158 (86.4)	20(10.9)	5 (2.7)	1 (0.5)	184 (100)

Table 4-14: Treatment outcomes in patients with SM+INH resistance according to different regimens

In patients with SM and INH resistant TB, if the regimen of 2SRHZ/6HE was used the cured rate was 76.7% (n=33/43) and the unfavourable outcome rate was 23.3% (n=10/43). In contrast, if the regimen of 2RHZE/6HE was used, the cured rate increased to 90.8% (n=118/130) and the unfavourable outcome rate decreased to 12.0% (n=12/130) (table 4-14). This difference was not statistically significant (OR=1.88[95 % CI 0.82-4.28] P=0.134).

4.3.11 Treatment outcomes of patients with INH monoresistance confirmed by LJ culture and DST

Fifty two cases with INH monoresistance diagnosed by LJ culture were investigated. In 52 patients with INH monoresistance by LJ, 48 cases were treated with the regimen of 2RHZE/6HE, 10 cases were treated with the regimen of 2SRHZ/6HE and 4 cases were treated with other individualized regimens (2SRHZ/SHE-Oflo/6E-Oflo, 2SRHZ/RHZ/5RHE, 3RHEZ/5HE and 3RHZE/3RH). Treatment outcomes were compared for patients receiving the two standardized regimens in 48 cases with INH monoresistant TB. Although the unfavourable outcome rate was doubled with 2SRHZ/6HE regimen (30.0%) compared to 2RHZE/6HE (15.8%) this difference was not statistically significant (P=0.370) among patients with INH monoresistance diagnosed by LJ culture (Table 4.15)

Regimen	**Favourable outcome** n (%)	**Unfavourable outcome** n (%)	**Total** n (%)	**OR, [95%CI] and p value***
2RHZE/6HE	32 (84.2)	6 (15.8)	38 (100)	OR=2.24 [0.29-14.12] p=0.370
2SRHZ/6HE	7 (70.0)	3 (30.0)	10 (100)	BASELINE
total	39 (81.3)	9 (18.8)	48 (100)	

Table 4-15 : Treatment outcomes in INH monoresistant TB patients (diagnosed by LJ) with two regimens

4.4 Discussion

4.4.1 Bacterial lineage

Beijing genotype was associated with young age, male sex, isoniazid resistance and MDR in this study. Indo-Oceanic strains were associated with older age and less likely to be either isoniazid resistant or MDR. Three major lineages of *M.tb* are circulating in Vietnam, those of the Beijing and Euro-American lineage belong to the 'modern' lineages as defined by deletion of the TD1 region, and the Indo-Oceanic linege is an 'ancient' lineage.

The evidence from this study contributes to the body of evidence that Beijing genotype strains are associated with drug resistance and with active transmission among young adults in Vietnam. Beijing genotype strains which were resistant to isoniazid were also more likely to carry a *KatG* mutation which confers a higher MIC than *inhA* or wild-type isoniazid resistant strains, whereas Indo-Oceanic strains were more likely to be wild-type with other unknown resistance mechanisms to isoniazid.

Overall, Beijing lineage was an independent risk factor for unfavourable outcome. The mechanism underlying this is unknown but may increase drug tolerance accross the spectrum of drugs used in multi-drug therapy due to an intrinsic mechanism such as efflux or permeability. Alternatively, Beijing genotype may subvert the immune response to *M.tb*, resulting in ineffective clearance of bacilli even after treatment is

initiated or have a higher tolerance to oxidative stress encountered within the phagosome [371].

Further research is required to elucidate the underlying mechanisms responsible for the increased treatment failure seen among patients infected with the Beijing genotype of *M.tb*. It would not be practical to screen patients for the infecting genotype, nor is it clear if alternative treatment regimens would be more effective as this will depend on the underlying mechanism responsible for these epidemiologically observed associations.

4.4.2 INH MIC

There was no association between high MIC to INH and unfavourable treatment outcome among patients with INH resistant TB. This suggests that INH MIC above 0.2 μg/ml are resistant to serum levels achieved under standard INH treatment doses and the current critical concentration for INH *in vitro* is appropriate. We were not able to evaluate pharmacokinetic levels of INH in patients which would have enabled us to examine the relationship between INH MIC and unfavourable outcomes in more detail. The influence of NAT2 acetylator status is investigated in the next chapter.

4.4.3 INH resistance mutation

The majority of INH resistant isolates in this study had a mutation in *katG* responsible for INH resistance (77.3%). This is consistent with data from other studies which shows a high prevalence of *katG* mutated isolates among INH resistant isolates from Vietnam and Asia [345-348] [349].

KatG mutation was significantly more likely to have high MIC to INH than other resistant strains. This is consistent with data from other studies and confirms that the *katG* mutation confers a high level of INH resistance. A study by Gagneux *et al.* also demonstrated that the backbone genotype of *M.tb* influences the level of resistance to INH conferred by a mutation, with Beijing genotype isolates carrying a *katG* mutation having a higher MIC to INH than *katG* mutated isolates of other lineages[177]. Again, the underlying mechanisms responsible for this association are unknown and require further investigation.

A recent study in the United States showed that patients with isoniazid resistant TB without *katG* mutation had a higher rate of sputum culture conversion at month 1 (aOR=4.4, 95% CI 1.1-23.6,p=0.04), which suggests *katG* mutants have a slower bacterial clearance in response to treatment [374]. A study conducted in Mumbai, India also showed an association between *katG* and poor outcome (p=0.037). However, in this study the GenoType MTBDR*plus* line probe assay was used to determine mutations and therefore only *katG* and *inhA* mutations were detected, rather than phenotypic resistance. Resistance to INH through *katG* mutation may also be associated with resistance to SM and EMB and this is likely to impact treatment outcomes [375] [376].

In the present study, there was no association between *katG* mutation and unfavourable treatment outcome in patients with INH resistance (non MDR) (aOR=0.59, 95% CI : 0.22-1.56, p =0.283) (Table 4-8 and Table 4-13). In South Africa a study also found no evidence of association between treatment outcome and specific isoniazid resistance conferring mutation performed by sequencing [331]. The

circulating backbone *M.tb* strains vary in different settings and this may account for the different findings. Global studies are required to fully elucidate the relationship between mutation, bacterial lineage and treatment outcome.

4.4.4 Impact of additional drug resistance on outcome

Patients with INH^R monoresistance had an unfavourable outcome of 21.2% compared with patients with INH^R +SM^R (10.9%), and patients with polyresistance (INH^R plus resistance to EMB or PZA, not MDR) (41.7%). Patients with additional drug resistance, excluding streptomycin, are likely to require treatment regimens including second line drugs, ideally a fluoroquinolone where susceptible. The optimal regimens and duration are undetermined and must be guided by patient treatment history, DST when available and response to therapy.

4.4.5 Conclusion

Beijing genotype was associated with INH resistance, MDR, young age and male sex. Among INH resistant isolates, Beijing genotype was also associated with unfavourable treatment outcome. No associations were identified between unfavourable treatment outcome and INH resistance mutation or MIC and unfavourable outcome. Further *in vitro* and epidemiological studies are required to understand the mechanisms responsible for the association of Beijing genotype and with treatment failure. These studies will improve understanding of the mechanisms responsible for the global emergence of the Beijing genotype and the evolution of mechanisms in *M.tb* which combat existing treating strategies and allow the emergence and propagation of drug resistant TB, which is a major threat to global TB control in the existing landscape of limited effective drug therapies.

Chapter 5

Drug-induced hepatitis and INH resistant TB

5.1 Aims

1) Determine the seroprevalence of hepatitis B surface antigen (HBsAg) and hepatitis C anti HCV in patients with INH resistant TB diagnosed by MODS
2) Characterise the incidence of symptomatic and asymptomatic ATDIH in patients with INH resistant pulmonary TB in Ho Chi Minh City
3) Identify risk factors associated with development of ATDIH.
4) Examine the effect of ATDIH on the viral dynamics of HBV in infected patients.

5.2 Introduction

Anti-tuberculous drug induced hepatitis or hepatotoxicity (ATDIH) is a potentially serious complication of the treatment of both active and latent tuberculosis with first line drugs. This hepatitis can be serious enough to result in death, although asymptomatic hepatitis (elevated liver transaminases in the absence of symptoms) is commoner. Pyrazinamide is the drug most frequently implicated, but hepatitis is also associated with other agents including isoniazid and rifampicin [377]. The risk of ATDIH is largely influenced by underlying risk factors and varies from 5 – 33% in published reports [378]. Interruption of treatment is often sufficient to resolve the hepatotoxicity. A gradual sequential re-introduction of each drug is usually well-

tolerated without recurrence of hepatotoxicity [292]. However, the overall outcome in TB is adversely influenced by treatment interruptions – for example, a trial of dexamethasone as adjunctive treatment in TB meningitis at our hospital found treatment interruption to be one of the most sensitive predictors of a poor outcome, after advanced disease stage at diagnosis. Treatment interruption is also associated with the development of drug resistance in TB [379]. Interestingly, in the study of dexamethasone in TB meningitis by our group there was a marked difference in the incidence of ATDIH between the patients receiving dexamethasone and those receiving placebo – there were no cases of severe hepatotoxicity in the steroid arm but 8/274 (2.9%, P=0.004) in the placebo arm, suggesting that the reduction in mortality in patients receiving steroids may in part be due to a hepato-protective effect of dexamethasone [380].

Factors that are implicated in the development of ATDIH include gender, older age, concomitant use of other hepatotoxic drugs, malnutrition, heavy alcohol consumption and pre-existing liver disease [281, 379, 381-385]. Racial and genetic factors (such as acetylator status) are also probably important although there is conflicting evidence over their roles [382, 386-388]. The role of factors such as co-infection with hepatotrophic viruses such as hepatitis B virus (HBV) and hepatitis C virus (HCV) is also unclear [303, 316, 381, 389]. Infection with these viruses is common in Vietnam, with HBV infection approaching 10% in the general population, and HCV infection rare in the general population but approaching 100% in some groups such as intravenous drug users [314, 390]. Similarly, the effect of ATDIH on the viral dynamics of these infections has not been studied. ATDIH may be associated with

increases in viral replication, which theoretically may have adverse consequences, since level of viraemia correlates with progression of liver disease. There are no data describing the relationship between the level of HBV or HCV viraemia and the subsequent risk of ATDIH. An understanding of the prevalence of ATDIH in Vietnamese patients and the ability to predict which patients are at risk of ATDIH would help in the design of clinical interventions to protect against ATDIH, which may have both a mortality benefit and a benefit in reducing the likelihood of TB drug resistance developing.

The aim of this chapter was to determine the seroprevalence of hepatitis B surface antigen (HBsAg) and hepatitis C anti HCV and to characterise the incidence of ATDIH in patients with INH resistant pulmonary TB in Ho Chi Minh City diagnosed by MODS, and identify risk factors associated with its development. The effect of ATDIH on the viral dynamics of HBV in infected patients was also examined.

5.3 Results

5.3.1 Prevalence of Hepatitis B/C infection among patients with INH^R TB

Among 347 patients with INH resistance by MODS who were tested for serum HBsAg and antiHCV at month 0, month 1, month 2 and month 3; 34 cases (9.8%) were seropositive for HBsAg in which there were 3 cases of seroconversion and 16 cases (4.6%) were positive for antiHCV with 2 cases of seroconversion. There was one case that was positive for both hepatitis B and hepatitis C infection. The total of patients with either hepatitis B or hepatitis C infection was 49/347 cases (14.1%).

5.3.2 Liver function tests in patients with viral hepatitis infection

Alanine aminotransferase (ALT) levels in patients infected with hepatitis B/C were as follows: mean 40.73 U/l, SD 75.74, range: 5-521. ALT level was elevated (>200 UI) in only 1 case (n=1/49, 2.0%). Mean pretreatment total bilirubin liver function tests in patients infected with viral hepatitis was 15.4 μmol/l (SD: 5.87, range: 2.2 -36). Nine patients (n=9/49, 18.4%) had elevated total bilirubin (above19 μmol/L). A single case of protein level was identified < 60 g/L (1/49 = 2%). For blood Albumin/Globulin ratio (A/G), there were 36 cases with A/G <1 (36/49 = 73.5%).

5.3.3 Demographic risk factors for viral hepatitis infection

Baseline demographic data for 34 patients with positive HBsAg are shown in Table 5-1.

	n (%)/ median [IQR]
Age	38 [28.8-43.5]
Male sex	29 (85.3%)
Occupation	
Manual labour	29 (85.3%)
Professional/administrative	1 (2.9%)
Retired/housewife	2 (5.9%)
No employment	2 (5.9%)
Total	34 (100%)
Duration of illness	30, [15-90 days]
Districts	
Pham Ngoc Thach	1 (2.9%)
Phu Nhuan	3 (8.8%)
District 6	4 (11.8%)
District 4	3 (8.8%)
Binh Thanh	4 (11.8%)
District 1	7 (10.6%)
District 8	4 (11.8%)
Tan Binh	8 (23.5%)
Total	34 (100%)

Table 5-1: Demographic characteristics of 34 patients with positive HBsAg test

Demographic characteristics of HBV infected and uninfected patients are compared in Table 5-2. No demographic factors associated with hepatitis B infection were identified.

Characteristics	HBsAg (+)	HBsAg (-)	Crude OR (95% CI)	p value
	n = 34	n = 313		
Age (median [IQR])	38 (29 - 44)	39 (28 - 48)	0.99 (0.97 – 1.02)	0.711
Male sex	29 (85%)	224 (72%)	2.30 (0.86 – 6.14)	0.095
Occupation: manual labour	29 (85%)	226 (72%)	2.23 (0.84 – 5.95)	0.108
Treatment centre: PNT*	1 (3%)	14 (4%)	0.65 (0.08 – 5.08)	0.679

*PNT compared with local DTUs

Table 5-2: Comparison of demographic factors among hepatitis B infected and uninfected patients

A total of 16 cases with positive antiHCV were identified. Baseline demographics for these patients are shown in Table 5-3.

	n (%)/ median [IQR}
Age	46 [35.0-52.5]
Male Sex	15 (93.8%)
Occupation	
Manual labour	9 (56.2%)
Professional/administrative	1 (6.3%)
Retired/housewife	3 (18.8%)
No employment	3 (18.8%)
Total	**16 (100%)**
Duration of illness	30 [14.0-67.5]
Treatment centre	
Pham Ngoc Thach	1 (6.3%)
Phu Nhuan	3 (18.8%)
District 6	4 (25.0%)
District 4	1 (6.3%)
Binh Thanh	3 (18.8%)
District 1	2 (12.5%)
District 8	1 (6.3%)
Tan Binh	1 (6.3%)
Total	16 (100%)

Table 5-3: Demographic characteristics of 16 patients with positive antiHCV test

5.3.4 Comparison of treatment outcomes in patients infected with hepatitis B/C and those uninfected

Of 49 cases positive for HBsAg or antiHCV, 9 cases were diagnosed as MDR and 3 cases received individualized regimens leaving 37 cases of hepatitis co-infection in the treatment outcome analysis. The treatment outcomes of this group were compared to patients negative for HBsAg and antiHCV among patients with INH^R (not MDR) TB.

Favourable outcome includes cured and treatment completed outcomes and unfavourable outcome includes died, lost to follow-up and treatment failed outcomes. There was no difference between treatment outcomes (favourable and unfavourable outcomes) in those infected with viral hepatitis and those uninfected OR=0.69 [95% CI 0.25; 1.89], P=0.475.The details are shown in Table 5-4.

	Favourable outcome n (%)	**Unfavourable outcome n (%)**	**Total n (%)**	**OR,[95% CI], p value***
HBsAG/ antiHCV negative	181 (80.1)	45 (19.9)	226 (100)	BASELINE
HBsAG/ antiHCV positive	29 (85.3)	5 (14.7)	34 (100)	0.69 [0.25-1.89], P=0.475
Total	210 (80.8)	50 (19.2)	260 (100)	

Table 5-4 : Association between treatment outcome and viral hepatitis infection

5.3.5 Incidence of antituberculous drug induced hepatitis

According to the diagnostic criteria for ATDIH (serum ALT levels > 3 times ULN plus clinical features of hepatitis such as anorexia, nausea and vomiting or serum ALT levels > 5 times ULN without symptoms and/or serum total bilirubin of > 32.49 μmol/L (> 1.5-1.7 times ULN with normal level of 19 μmol/l) [378], there were 11 cases with ATDIH. The cumulative incidence risk of DIH was 3.17% (11/347). Based on the World Health Organization (WHO) Toxicity Classification Standards [140, 391], there was one case with mild DIH (ALT at month 1: 181 U/l) (mild ATDIH is defined as ALT elevation of 3-5 times the ULN (121-200 U/l), 6 cases were considered as moderate DIH (5-10 times the ULN: 201-400 U/l) and no case with severe ATDIH (> 10 times the ULN : > 400 U/l). There were 4 cases with increased serum bilirubin (two cases at month 2 and two cases at month 3). One patient developed the increase of ALT at month 1, three cases developed the increase of ALT at month 2 and three cases increased ALT levels at month 3. Six cases (3 cases with positive HBsAg tests and 3 cases with positive antiHCV tests) developed ATDIH (the rate for DIH in patients with viral hepatitis B/C was 6/49 = 12.2%). All these 4 cases did not have increase in Akaline Phosphatase (ALP) (The normal value of ALP ranges from 50 to 258 U/l).

5.3.6 Risk factors for ATDIH

No significant differences were observed in terms of age, gender, serum protein, BMI, between patients who had ATDIH and those who did not have ATDIH (Table 5-5). Patients with hepatitis B infection were significantly more likely to have ATDIH than those without viral hepatitis infection (37.5% vs. 9.3%) OR=5.86 [95% CI 1.33-25.7], P=0.019. Patients with hepatits C infection were also at significantly greater risk of ATDIH than those without viral hepatitis infection: OR=14.7 [95% CI 3.13-68.6], P=0.001. Overall viral hepatitis infection (B/C) was significantly associated with ATDIH OR=8.18 [95% CI 2.39-28.0], P=0.001. The details are shown in Table 5-5.

Risk factor N=2,090	Crude OR (95% CI)	p value
Age* (years)	1.02 (0.98, 1.05)	0.378
male gender n=1524	1.06 [0.28-3.99	0.937
HBsAg (+) n=34	5.86 [1.33-25.7]	0.019
HCVAb (+) n=16	14.7 [3.13-68.6]	0.001
HBsAg (+) / HCVAb (+) n=49	8.18 [2.39-28.0]	0.001
Serum protein (g/L)	1.01 [0.91- 1.12]	0.838
BMI (kg/m^2)	1.06 [0.84- 1.34]	0.634

Table 5-5 : Risk factors for ATDIH

5.3.7 Treatment outcomes in patients with ATDIH

Treatment outcomes were compared between patients with and without ATDIH, excluding MDR cases and those treated with individualized regimens.

There was no significant difference in treatment outcomes (unfavourable and favourable outcomes) between patients with ATDIH and those without ATDIH for patients with INH resistant TB (non-MDR). The number of ATDIH events in the study overall was too low to detect a significant difference (Table 5-6).

	Favourable outcome n (%)	**Unfavourable outcome n (%)**	**Total n (%)**	**OR, 95%CI, p value**
No ATDIH	204 (80.6)	49 (19.4)	253 (100)	BASELINE
ATDIH	6 (85.7)	1 (14.3)	7 (100)	0.69 [0.82; 5.90], 0.738
Total	210 (80.8)	50 (19.2)	260 (100)	

Excluding MDR and patients receiving individualized regimens.

Table 5-6: Treatment outcomes among patients with ATDIH and without ATDIH (patients with INH^R not MDR)

5.3.8 Viral dynamics in patients infected with viral hepatitis

There were 34 cases with positive HBsAg in which 31 cases (3 cases were not measured HBV DNA) HBV DNA was measured at month 0, month 1, month 2 and month 3. In one case HBV DNA was not detected at month 0, month 1, month 2 and month 3. There were 18 cases in which HBV DNA was detected at month 0, month 1, month 2 and month 3. In seven cases HBV DNA was detected at 3 times. In two cases HBV DNA was detected at 2 times and three cases HBV DNA was detected only one time. The values of HBV DNA were transferred to logarithm of 10.

Figure 5-1 shows HBV DNA viral load counts of 30 patients related to month 0, month 1, month 2 and month 3.

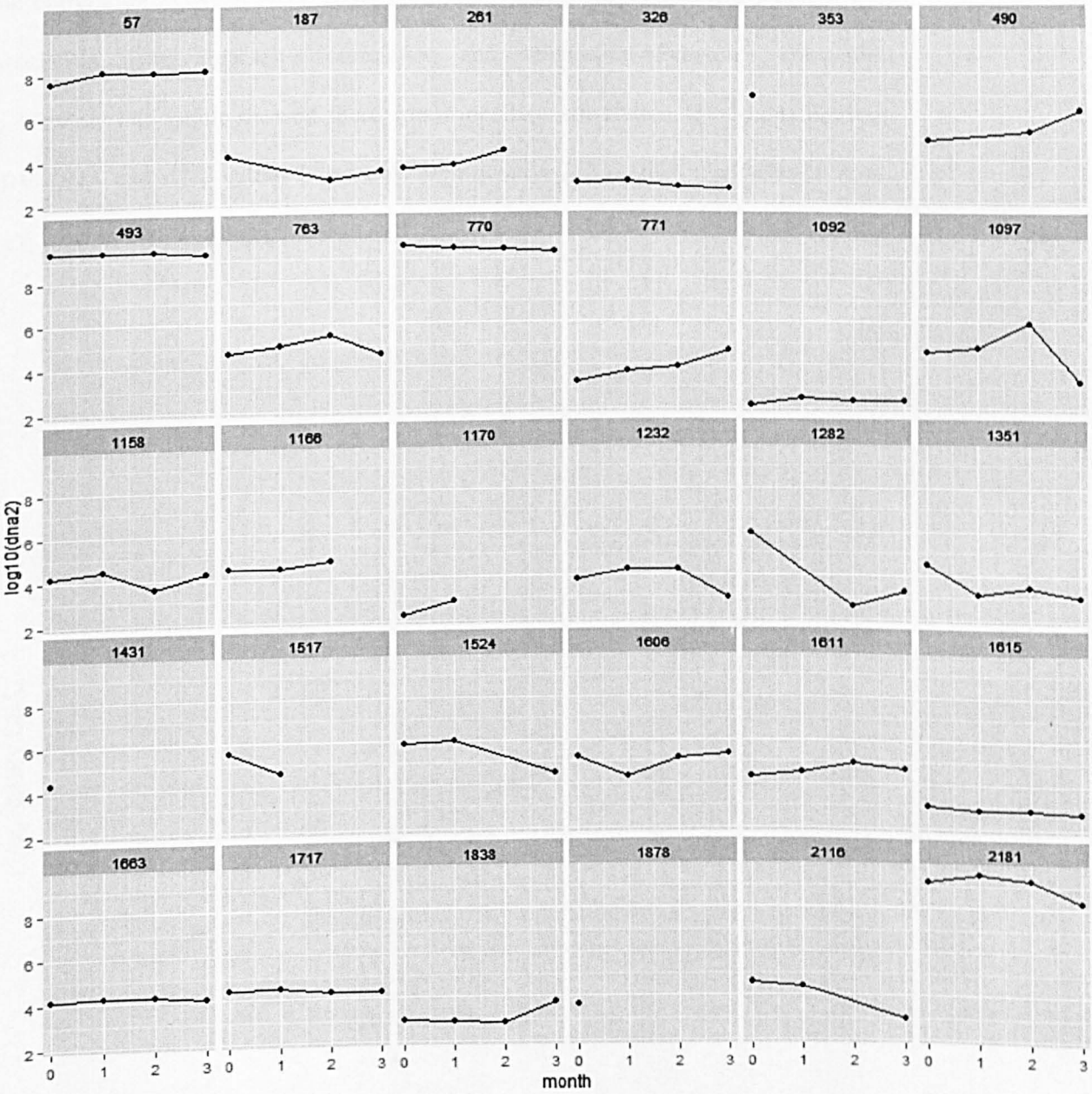


Figure 5-1: Dynamics of HBV DNA counts at month 0, 1, 2 and 3 in 30 patients with positive HBsAg tests

The correlation between HBV DNA and ALT values was evaluated by measure of association using Pearson's correlation and calculating Pearson's correlation coefficient. The result showed there was a positive correlation (r = 0.24) between HBV DNA and ALT values in patients with positive HBV DNA test (95%CI: 0.04-0.42, p= 0.02) (Figure 5-2).

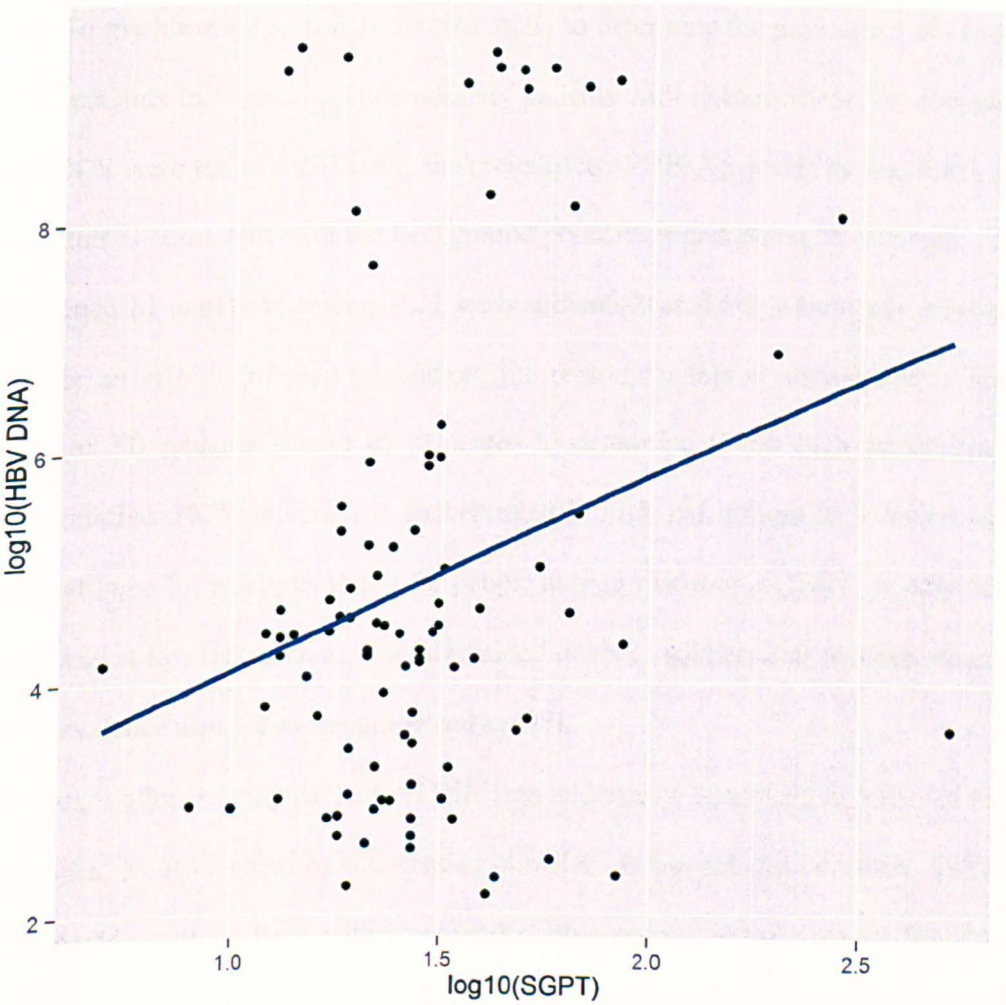


Figure 5-2 : Association between HBV DNA and ALT values in patients with positive HBV DNA

5.4 Discussion

5.4.1 Prevalence of viral hepatitis among Vietnamese INH^R TB patients

Hepatitis B virus infection is common in Southeast Asia and the Western Pacific with a prevalence of from 2% to 31% [392, 393]. Studies in different regions of Vietnam have shown a prevalence of HBsAg of between 4 and 18% in the population[394-396]. To my knowledge, this is the first study to determine the prevalence of HBsAg in TB patients in Vietnam. Although only patients with INH resistant TB diagnosed by MODS were tested for HBsAg, the prevalence of HBsAg positivity was 9.8% and this result is consistent with the background population prevalence in Vietnam. The prevalence of antiHCV among INH study patients was 4.6% which was relatively high for an HIV uninfected population; the reason for this is unclear and a larger group of TB patients should be evaluated to determine if this high prevalence is representative. HCV infection is concentrated in high risk groups in Vietnam as in other settings; for example 55.6% for people at high risk such as IDUs, or only 0.5% for people at low risk such as voluntary blood donors, soldiers and pregnant women. The prevalence also varies geographically [397].

Treatment after development of ATDIH was undertaken according to Vietnam NTP guidelines, as determined by the treating clinician. When patients exhibited ATDIH, all hepatotoxic drugs were withdrawn and nonhepatotoxic drugs such as SM, EMB and quinolone were used. After liver function (ALT, bilirubin) returned to normal, the hepatotoxic drugs were reintroduced sequentially for each drug at standard doses (RiF and INH and PZA), one per week. If symptoms of ATDIH and/or liver function tests

increased during the reintroduction, the offending drug was confirmed and then withdrawn from the regimen.

5.4.2 Risk factors for ATDIH in Vietnamese TB patients

In the current study, patients coinfected with hepatitis B/C were at much greater risk of ATDIH compared to patients without Hepatitis B/C infection (OR=8.18 [95% CI 2.39-28.0], P=0.001)). HBV/HCV infected patients were not more likely to have unfavourable outcomes (OR=0.98 [95% CI 0.41-2.38], P=0.972) possibly due to early detection and experienced clinical management.

There was no significant association between ATDIH and demographic factors of age, gender, and BMI in this study. The reasons for lack of observed associations with risk factors described in other populations are probably due to the low number of ATDIH events, limiting the power to detect associations.

The risk of ATDIH during treatment of TB patients who are seropositive for the hepatitis C virus (HCV) has been investigated. A study from Korea showed that ATDIH occurred more commonly in HCV-seropositive patients compared to control group, although the difference was borderline statistically significant with p value of 0.056 [398]. In contrast, a study from Taiwan showed HCV co-infection had a high incidence of ATDIH during TB treatment (OR 3.43, [95%CI 1.14-10.35], P = 0.03) [315]. This study advised screening for HCV infection before TB treatment in order to determine follow-up monitoring. In the present study, HCV infection was also a strong risk factor for ATDIH (OR=14.7 [95% CI 3.13-68.6], P=0.001) although only 16 HCV infected patients were identified. The nature of HCV disease may also be a

confounding factor because liver function tests (ALT) in patients HCV seropositive may be normal or wax and wane over time [316].

Chronic hepatitis B infection has been reported as a risk factor for ATDIH elsewhere [389]. For HBV carriers, a study from China showed the incidence of abnormal liver function tests was higher in HBV carriers (34.9%) on TB therapy compared to HBV noncarriers (9.4%) on TB therapy ($p<0.01$) and to HBV carriers (8.1%) not given any anti TB drugs ($p<0.01$) [303]. The theory suggested that anti TB drugs may have a direct effect in hastening HBAg seroconversion [303]. However, there are no studies demonstrating a difference in liver damage due to ATDIH and hepatitis B viral activity.

5.4.3 Impact of ATDIH on treatment outcomes

There was also no difference in treatment outcomes between INH^R patients (not MDR) with ATDIH and those without ATDIH which may be due to the small number of ATDIH events (7 cases with DIH were analysed). A larger study would be required to have sufficient power to detect an impact of ATDIH on treatment outcomes.

5.4.4 Correlation of HBV viral load and liver function

Higher HBV DNA values correlated with increased ALT values with correlation coefficient of 0.24 [95%CI: 0.04-0.42], p= 0.02). This result demonstrates the underlying association between higher viral load and ATDIH in patients with TB and on TB treatment.

5.4.5 Study limitations

The study had some limitations. The criterion for hyperbilirubinemia was a bilirubin level > 1.7 times the ULN. In patients with this criterion, hyperbilirubinemia may be due to adaption or a transient increase and so it is possible that some patients without true ATDIH were included. The number of ATDIH cases occurring during the study was low (11 cases) and it was not possible to examine Hepatitis B/C infection and ATDIH incidence in all patients included in the screening study due to resource limitations, which would have given greater power to detect risk factors for both Hepatitis B/C infection and ATDIH.

5.4.6 Conclusion

In summary, the study showed that in patients with INH resistant TB diagnosed by MODS, the prevalence of HBsAg positivity was 9.8% and antiHCV positivity was 4.6%. The cumulative incidence risk of ATDIH was 3.17%. Viral hepatitis infection was strongly associated with ATDIH (OR=8.18, [95%CI 2.39-28.0], p= 0.001) but not with unfavourable outcomes. Ideally, Hepatitis B vaccination programmes should be applied to reduce the prevalence of hepatitis B infection in the population. In the meantime, this data emphazises the need for screening of hepatitis B/C infection among TB patients prior to initiation of TB therapy to identify patients at high risk for ATDIH and requiring more intensive monitoring for liver function, especially in populations with a high prevalence of viral hepatitis. Resource limitations may necessitate targeted Hepatitis B/C screening and further study is required to identify the population-based risk factors for Hepatitis B/C infection in the Vietnamese population.

Chapter 6

N-acetyltransferase-2 (NAT2) genotype and treatment outcomes of INH resistant TB

6.1 Aims

1) Determine the prevalence of predicted fast acetylator phenotype in Vietnamese TB patients by NAT2 genotype sequencing.
2) Determine the impact of predicted fast acetylator phenotype on treatment outcomes in patients with INH-resistant TB by INH MIC level.

6.2 Introduction

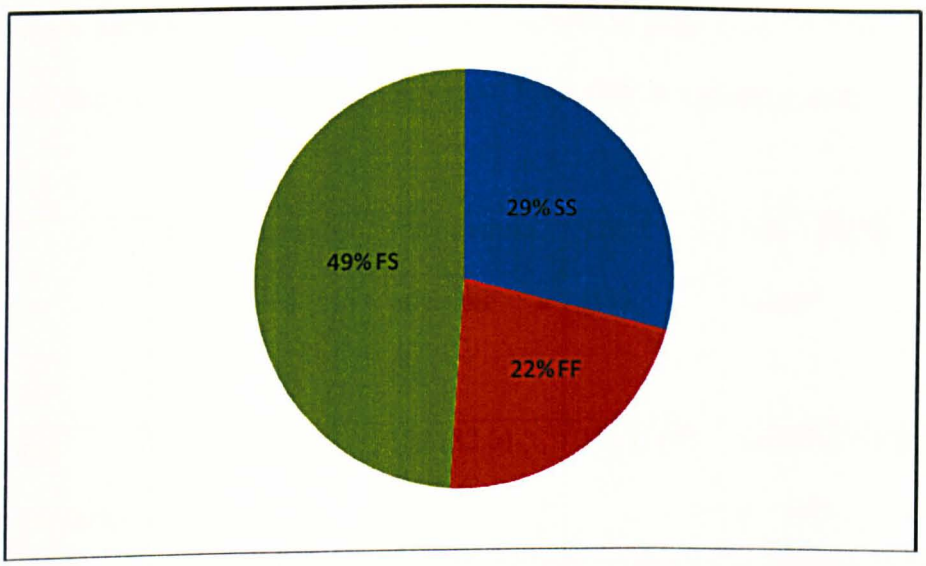
Isoniazid is eliminated by the N-acetyltransferase-2 (NAT2) enzyme system. Individuals are either homozygous rapid acetylators (FF), heterozygous (FS), or homozygous slow acetylators (SS) [237]. It is known that NAT2 genotype affects the serum concentration of isoniazid, influencing the Early Bactericidal Activity (EBA) of isoniazid which is optimal in patients with fully sensitive isolates at 2-3μg/ml serum concentration [237, 243]. The NAT2 genotype therefore may also impact on the efficacy of isoniazid therapy in patients infected with isolates with a slightly raised MIC. The Vietnamese are likely to be predominantly fast acetylators as

Chinese, Japanese and Thai ethnic groups are predominantly fast acetylators, in contrast to Caucasian and African populations, where the majority are slow [248, 249] We therefore determined the acetylator genotype assigned on the basis of 6 known SNP's in the NAT2 gene (C190T, G191A, T341C, C481T, G590A and G857A) in the Vietnamese population and evaluated the role of predicted NAT2 fast acetylator phenotype on TB treatment outcomes in patients infected with *M.tb* with high or low level resistance to isoniazid.

6.3 Results

6.3.1 Distribution of acetylator phenotypes predicted by genotype among patients with INH-resistant TB

260 cases with INH^R (not MDR) were sequenced to determine the acetylator genotype. The results showed 58 cases (22%) were predicted to be homozygous rapid acetylators (FF), 126 cases (49%) were heterozygous (FS) and 74 cases (29%) were homozygous slow acetylators (SS). One case was determined as unknown acetylator genotype and one case was missing for NAT2 genotype (Figure 6-1).



FF= fast acetylator
FS=Intermediate acetylator
SS= slow acetylator

Figure 6-1 : The distribution of different acetylator genotypes in 258 cases with INH^R (not MDR)

6.3.2 Treatment outcomes by predicted acetylator phenotype among patients with INH-resistant (non MDR) TB

The treatment outcomes determined by culture in addition to smear, categorized as favourable or unfavourable, of 260 patients with INH^R (not MDR) were analyzed by predicted acetylator phenotype. Two cases were excluded from the analysis because of missing or indeterminate genotype. The predicted acetylator phenotypes were divided into 2 groups including 184 cases (71.3%) with fast acetylator (FF and FS) and 74 cases (28.7%) of slow acetylator (SS) phenotype. The results of treatment outcomes are presented in the Table 6-1. Overall, there was no significant difference

between the treatment outcomes by predicted acetylator phenotype (P=1.00). Indeed the proportion of unfavourable outcomes was 17% in both acetylator groups.

	Unfavourable outcome n (%)	**Favourable outcome n (%)**	**Total n (%)**	**OR, [95%CI], p value***
Fast acetylator	32 (17.4)	152 (82.6)	184 (100)	0.99 [0.47-2.20], p = 1.00
Slow acetylator	13 (17.6)	61 (82.4)	74 (100)	BASELINE

Table 6-1 : Treatment outcomes in INH^R patients (not MDR) with fast and slow acetylators

6.3.3 Treatment outcomes in fast acetylator patients infected with M.tb with low-level INH- resistance

The association between predicted acetylator phenotypes (fast and slow) and treatment outcome in patients infected with isolates with high MIC to INH (MIC $\geq$1 µg/ml) versus low MIC (0.2 µg/ml $\leq$ MIC < 1 µg/ml) was further investigated. Patients who were MDR (n=54) or received individualized regimens (n=16) were excluded, leaving 239 patients in the analysis. There were 154 cases (64.4%) who were fast acetylators infected with isolates with high MIC, 55 cases (23.0%) who were slow acetylator and high MIC, 19 cases (8.0%) with fast acetylator and low

MIC, and 11 cases (4.9%) with slow acetylator and low MIC. In theory, patients who are fast acetylators and infected with isolates with low MIC might have unfavourable outcomes compared with slow acetylator patients infected with isolates with a low MIC. We compared the treatment outcomes in these groups. The results are shown in Table 6.2.

NAT2	MIC	Favourable outcome n (%)	Unfavourable outcome n (%)	Total n (%)
Fast	**Low**	15 (79.0)	4 (21.1)	19 (100)
Slow	**Low**	9 (82.8)	2 (18.2)	11 (100)
Slow	**High**	46 (83.6)	9 (16.4)	55 (100)
Fast	**High**	126 (81.8)	28(18.2)	154 (100)
Total		196 (82.0)	43 (18.0)	239 (100)

MDR and patients receiving individualized regimens excluded

Table 6-2 : Treatment outcomes by acetylator status and INH MIC level among patients with INH^R TB

NAT2	MIC	Crude OR			Adjusted OR*		
		OR	95% CI	P-value	OR	95% CI	P-value
Fast	Low	BASELINE					
Slow	Low	0.83	0.13-5.50	0.850	0.78	0.11-5.29	0.797
Slow	High	0.73	0.20-2.73	0.644	0.72	0.18-2.85	0.638
Fast	high	0.83	0.26-2.73	0.761	0.82	0.25-2.77	0.753
All groups compared to baseline		0.81	0.25-2.57	0.718	0.80	0.24-2.63	0.709

*Adjusted for streptomycin, ethambutol and pyrazinamide resistance and treatment regimen.

MDR and patients receiving individualized regimens excluded.

Table 6-3: Risk of unfavourable outcome by acetylator status and INH MIC

There was no significant difference between unfavourable treatment outcomes among patients with fast acetylator status infected with low MIC isolates compared with others (OR=0.80 [95% CI 0.24-2.63], P=0.709) (Table 6-3).

6.3.4 Impact of acetylator phenotype on ATDIH risk

Patients with a predicted slow acetylator phenotype were significantly more likely to develop ATDIH (OR=4.62 [95% CI 1.17-18.3], P=0.029). This remained true when adjusted for viral hepatitis infection (OR= 6.23 [95% CI 1.46-26.49, P=0.013) (Table 6-4).

NAT2	No ATDIH	ATDIH	Crude OR			Adjusted OR*		
			OR	95% CI	P-value	OR	95% CI	P-value
Slow	93	7	4.62	1.17-18.3	0.029	6.23	1.46-26.49	0.013
Fast	184	3	BASELINE					

*Adjusted for viral hepatitis infection.

Table 6-4: Impact of acetylator status on risk of ATDIH in patients with INH resistant tuberculosis.

6.4 Discussion

This study confirmed there is a predominance of fast acetylators (71.3%) for isoniazid among the Vietnamese population. During the first 2 to 5 days of TB treatment, INH drives the bactericidal activity of the regimen. PZA is effective against slow growing TB bacilli and RiF is effective against nonreplicating TB bacilli. EBA of isoniazid is influenced by the NAT2 genotype. A study conducted in South Africa found that, heterozygous fast (FS) and homozygous fast (FF) acetylators had a lower EBA compared to homozygous slow (SS) acetylators with a standard isoniazid dose [131]. A maximum EBA of INH was reached with a serum concentration of INH of 2-3 μg/ml 2 hours post-dose. Therefore, increasing the dose of INH would increase the INH concentration but would not increase the EBA. For rapid and slow acetylator, the mean elimination half-lives of INH are 80 mintutes and 180 minutes, respectively.

There was no detected association of acetylator status with unfavourable outcome for patients with isoniazid resistant TB in the study reported here and this was also true when patients were stratified by MIC to INH of the infecting isolate. Data presented in chapter 4 showed that INH MIC alone was not a significant risk factor for unfavourable outcome and the data presented here suggests that this is not influenced by acetylator status of the patient, although the study may have been too small to detect an association. We were not able to examine isoniazid serum pharmacokinetics in this study which would have confirmed the predicted acetylator phenotype of patients and provided more reliable data on the serum INH levels achieved, which can be influenced by many factors including nutrition and co-morbidities. However, this data suggests that programmatic screening for acetylator phenotype alone among

patients with INH resistant TB would not be useful in determining which patients may benefit from increased INH dose or be at risk of treatment failure on standard regimens.

A larger study of acetylator phenotypes among TB patients would establish if there is any association with unfavourable outcome among patients with drug susceptible TB. Compared to rapid acetylators, slow acetylators have been reported to be more likely to develop polyneuropathy and drug-induced hepatotoxicity (ATDIH) [399, 400]. [401, 402]. Among Vietnamese TB patients, slow acetylators were also significantly more likely to develop ATDIH in this study (OR=6.23 [1.46-26.49]) which suggests a rapid test for determination of acetylator status may have clinical value. No such commercial test currently exists. Alternative strategies could be used to develop such a test, including determination of INH serum level at 2 hours post-dose or rapid genotyping using pyrosequencing or PCR-RFLP. Low plasma isoniazid concentration in rapid acetylators is considered one of many reasons for failure in TB treatment with the standard regimen (5 mg/kg of isoniazid) [403, 404]. However, in general, with standard daily isoniazid treatment, both rapid and slow acetylators have the same treatment efficacy [405, 406].

Analysis of pharmacokinetics of isoniazid among 18 Caucasian healthy volunteers in Germany showed substantial variation in isoniazid exposure which was largely explained by NAT2 variation. The authors recommended adjustment of the dose of isoniazid to 2.5 mg/kg, 5.0 mg/kg and 7.5 mg/kg for patients with none, one and two rapid NAT2 alleles, respectively [246]. A clinical trial from Japan randomized 172 patients to either standard or NAT2-guided INH dose. Low, medium or high dose

INH were used for slow, intermediate or fast acetylators repectively. The trial showed that the incidence of ATDIH was substantially lower in slow acteylators given a low dose (2.5mg/kg) of INH compared to those receiveing a standard dose (5mg/kg) 78% vs. 0% without apparent impairment of treatment efficacy. On the other hand, rapid acetylators, who received high dose of isoniazid (7.5mg/kg), achieved a lower rate of early clinical failure (15 vs. 38%) compared to the patients who were on standard treatment [407] . This trial supports systematic screening for NAT2 genotype and tailored INH dosage. However, at the present time this approach is not feasible in developing world settings and would require development of a rapid inexpensive point-of-care test. Further research would be needed to demonstrate a consistent benefit of treatment stratification across all ethnicities to justify the implementation of such an approach programmatically.

Chapter 7

Discussion

Globally, the prevalence of INH resitance among *M.tb* is increasing which presents a serious challenge to the target of TB elimination by 2050. Isoniazid is one of only six truly effective drugs for the treatment of TB, alongside rifampicin, streptomycin, ethambutol, pyrazinamide and fluoroqinolones. Each TB drug has a different spectrum of activity and INH is the key drug in rapidly reducing bacterial load and therefore infectiousness in the first days of treatment. Rifampicin is active against non-replicating bacilli and is the key companion drug in clearing INH resistant or tolerant persisters. Pyrazinamide has a unique spectrum of activity at low pH and is active against bacteria with low metabolic activity and therefore plays the crucial role in sterilizing persistent bacilli. Ethambutol and streptomycin are weaker anti-tuberculous agents and their principal role in multi-drug therapy is to guard against the emergence of resistance to the stronger agents and to maintain treatment efficacy in patients with undiagnosed resistance to other drugs within the regimen. The later generation fluoroquinolones (levofloxacin, moxifloxacin and gatifloxacin) have an EBA similar to that of isoniazid but it is unclear if regimens which substitute a FQ for INH will have similar efficacy. Treatment trials are underway which are testing if FQ can allow shorter TB treatment regimens of four months rather than six OFLOTUB NCT00216385[408], REMOX TB NCT00864383. However, widescale use of FQ in programmatic standardised regimens would likely lead to an increased prevalence of

FQ resistance and reduce the potential of FQs to treat INH and MDR resistant TB. The approval of bedaquiline for MDR TB [22]was a landmark in the development of antituberculous drugs but the pipeline remains extremely weak and new regimens containing multiple novel drugs are urgently required in order to address the epidemic of drug-resistant TB. A major challenge to global TB control programmes in the next decade will be to balance the need for individual access to life-saving regimens while preserving efficacy for communities. By developing and approving fixed-dose combination regimens of novel agents, there is an opportunity to develop a model for implementation of new drugs for infectious diseases which enables access while preserving susceptibility, but this remains a daunting challenge.

7.1 Impact of INH resistance on treatment outcomes

The unfavourable treatment outcome rate of INH resistant TB (19% at treatment completion) shown in this study coupled with the high prevalence of INH resistant TB in Vietnam, underlines the urgent need for trials determining the optimal treatment strategy for INH resistant TB. While systematic review data suggests additional drugs and/or prolongation of treatment are effective strategies for achieving high treatment success rates, all guidelines are based on expert opinion and no high-quality trial data exists to guide National TB Programmes in deriving standardized recommendations. While the implementation of drug susceptibility testing for every TB patient remains an impractical ideal, national TB programmes in countries with a high prevalence of INH resistant TB must consider implementation of standardized regimens which have improved efficacy for undiagnosed INH resistant TB while not increasing toxicity to unacceptable levels for those who are receiving additional unnecessary drugs. The

current WHO recommendation of an HRE continuation phase remains untested and data from Vietnam will be crucial in determining the effectiveness of this strategy [129].

7.2 Bacterial risk factors for unfavourable outcome among patients with INH resistant TB

This thesis addressed a number of potential risk factors for poor treatment outcome among patients with INH resistant TB to determine if a screening strategy could assist in detecting those at high risk of treatment failure on standardized regimens and to prioritise these patients for alternative interventions which may present an alternative to changing the standardized regimen for all patients. Bacterial mutation responsible for INH resistance was not a risk factor for unfavourable outcome although *katG* mutations were associated with higher MIC to INH. This indicates that the molecular line probe assays which determine the mutation responsible for INH resistance cannot be used to identify those at higher risk of unfavourable outcome.

Beijing genotype is independently associated with unfavourable outcome among patients with INH resistant TB and this finding requires further investigation to identify the underlying mechanism responsible. There is a growing body of evidence that Beijing genotype is associated with increased drug resistance and, given the epidemic emergence of the Beijing genotype in some regions, a greater understanding of this phenomenon is needed to avert the further propagation of MDR and XDR strains of TB. It is important to note that studies in several regions have failed to find any association of Beijing genotype with drug resistance and this may be due to differences in the circulating background strains to which Beijing genotype is

compared, sub-lineages of the Beijing genotype which have a propensity to develop resistance rather than the whole clade, programmatic differences in the management of TB, or to differences in the host genetics of the population which may influence the ability of Beijing genotype to subvert the immune response. Whole genome sequence analysis, host susceptibility studies coupled with bacterial sequencing, *in vitro* and *in vivo* immune studies and novel bacterial resistance models will all help to elucidate the mechanisms which Beijing genotype has evolved to evade drug therapy.

7.3 Patient risk factors for unfavourable outcome from INH resistant TB

Acetylator status and co-infection with viral hepatitis were investigated for association with unfavourable outcome among patients with INH resistant TB. No association was found. Although patients with viral hepatitis (B/C) were at strongly increased risk of drug induced hepatotoxicity, these patients did not have a higher risk of unfavourable outcome. This suggests that, despite the high prevalence of viral hepatitis among Vietnamese TB patients, the existing protocols for management of ATDIH are adequate but this should be interpreted with caution due to the low number of events. This may vary from other settings where there is a lower prevalence of viral hepatitis and less experience in the management of ATDIH.

Pharmacokinetics and EBA studies have shown that acetylator status is important in determining serum isoniazid levels achieved on standard INH doses but it is unclear the extent to which this affects treatment outcomes and if acetylator status determination would be of value in programmatic management of TB. This thesis investigated outcomes only among INH resistant TB patients and acetylator status may be an important determinant of outcome for patients infected with fully

susceptible isolates, but these patients generally achieve very high treatment success rates on standardized therapy and the cost of acetylator status screening is unlikely to be justified. The data presented in this thesis indicates that acetylator status screening will not assist in identifying those at increased risk of treatment failure among patients with INH resistant TB.

7.4 Recommendations for further research

1) Determination of all INH resistance mechanisms in *M.tb*

Resistance to INH in *M.tb* is not fully understood, more than fifty years after its discovery. While the proportion of *M.tb* isolates carrying known INH resistance mechanisms varies geographically, it is clear that the resistance mechanism is undetermined in 10-20% of phenotypically resistant *M.tb* isolates. Whole genome sequencing projects have provided clues to alternative mechanisms of resistance but these are yet to be conclusively validated and it is likely that efflux pump or other mechanisms also play a role, particularly in low-level but clinically important resistance. The lack of comprehensive knowledge of INH resistance mechanisms means that genotypic tests for the detection of INH resistant TB have limited sensitivity and *in vitro* research is required both to confirm postulated mechanisms of resistance and determine the role of novel mechanisms.

2) Development of a point-of-care test for INH resistant TB

There has been a huge global investment in the development of point-of-care tests for TB in the last decade, with a focus on TB detection and MDR detection. While this has yielded some impressive advances, most notably with the development and application of the Xpert MTB/RIF assay, an ideal point-of-care test which can be applied at TB clinics remains elusive. Phenotypic methods for the detection of TB and drug resistance, such as MODS, have been evaluated and endorsed by WHO, but are challenging for programmatic application due to the resource and biosafety requirements. Similarly, line-probe assays which can detect INH resistant TB have prohibitive costs which prevent application to all TB patients.

The global increase in INH resistant TB, the important role of INH resistant TB in fuelling the development of MDR TB and the evidence, including that in this thesis, that INH resistant TB has unacceptable treatment outcomes using current strategies, all underline the urgent need for a point-of-care test for INH resistant TB which can be applied programmatically. The current development pipeline for novel TB diagnostics does not include any tests for INH resistant TB which is likely to be scaleable to all TB patients.

3) Randomised controlled trials to determine optimal treatment for INH resistant TB

WHO has recognized the limited evidence base to inform the treatment of INH resistant TB and currently recommends countries with a 'high' prevalence of

INH resistant TB implement a HRE continuation phase, based on expert opinion. Alternative strategies would be to use a fluoroquinolone in addition to or to replace INH in these settings, or to prolong treatment. The widespread use of a fluoroquinolone in standardized regimens would carry a high risk of propagating fluoroquinolone resistant strains. Fluoroquinolones remain the most effective drug in the treatment of MDR TB and therefore the consequences of increased fluoroquinolone resistance are grave in the absence of alternative regimens for MDR TB. High quality randomized controlled trials are required to determine if the HRE continuation phase is effective for INH resistant TB and if the use of this regimen in all patients increases the incidence of ocular toxicity. Comparative trials of alternative treatment regimens would determine the most effective standardized regimen for INH TB. These should include assessment of adverse events and health economics modeling to determine which regimens should be applied in national TB programs.

4) Evaluation of cost-effectiveness of screening and treatment strategies for INH resistant TB

Modelling of the long-term cost-effectiveness of alternative screening and treatment strategies for INH TB are required to help National TB programmes determine the true costs of both implementation and neglect of INH resistant TB. The failure to address the increasing prevalence of INH resistant TB globally is creating a reservoir of drug resistant TB which, when ineffectively treated under current strategies, leads to the development and propagation of

MDR TB. While detection and treatment of MDR TB is being scaled up globally, there is no evidence-based strategy to address INH resistant TB. The increased application of INH prophylaxis, particularly in regions of high HIV prevalence, is likely to lead to increase in INH resistant TB in areas which currently have relatively low rates. While the costs of any screening strategy for INH resistant TB are likely to be substantive, improved data on true treatment failure rates for INH resistant TB and the impact on MDR TB rates will allow *in silico* models to more accurately determine the potential impact of screening and treatment strategies on the epidemic and the potential contribution towards the ultimate goal of TB elimination by 2050.

5) Elucidation of the underlying mechanisms responsible for the association of Beijing genotype with drug resistance and unfavourable treatment outcome

It has become clear in the last decade that there are substantive differences in *M.tb* genotypes, which were previously thought to be highly clonal, largely due to the focus of research in the last century on strains from Europe and America. The increased application of advanced research techniques to global collections of clinical *M.tb* strains has revealed the global population structure of *M.tb* but we have yet to understand the forces driving *M.tb* evolution and how these variations affect transmission and clinical disease. It is now clear that the long co-evolution of *M.tb* with human host populations of different ethnicities has resulted in local co-adaptation and there consequently exists variation in the interaction between different *M.tb* lineages and human host

immune responses. Human host susceptibility to *M.tb* infection has been studied in isolation from the variation in *M.tb* strains and the field of research into host pathogen interaction in TB has gained momentum only in recent years. It has been hypothesized that rapid urbanization of human populations has driven modern *M.tb* lineages such as the Beijing genotype towards a phenotype which favours active disease rather than latency. Further research is required to prove this hypothesis. Variations in immune interaction may have important implications for vaccine development, as a vaccine that is effective in one region may be less effective in regions with a predominace of different *M.tb* lineages or different ethnic human populations. It is therefore important to conduct both epidemiological and *in vitro* research into the mechanisms responsible for the association of Beijing genotype with treatment failure.

The association of Beijing genotype with drug resistance also requires further investigation to elucidate the underlying mechanisms. It is important to understand if the Beijing genotype has an intrinsic propensity for resistance which will apply to novel agents as they become available, or if the mechanism is specific to certain drugs. Proposed mechanisms of resistance include increased mutational frequency or compensatory mechanisms which allow the Beijing genotype to tolerate resistance mutations with a lower fitness cost. *In vitro* evidence has been found for both strategies but remains to be confirmed in larger studies. Whole genome sequencing projects of global *M.tb* collections of both drug resistant and drug sensitive strains will also contribute

to the understanding of mechanisms of drug resistance in *M.tb* and the isolates collected in this study are being sequenced.

The momentum of the millennium development goals has led to impressive advances in TB research after decades of stagnation. The pace of progress must be maintained, and indeed, increased, if we are to achieve the goal of TB eradication by 2050. In order to stem the surge of MDR TB it is crucial to understand the fundamental mechanisms of INH resistance in *M.tb*, develop rapid point-of-care diagnostics and optimized regimens for the treatment of INH^R strains. The need for multi-drug therapy in TB and the extremely long time required from drug discovery to approval will ensure that INH remains the most essential antituberculous drug, alongside rifampicin, for the foreseeable future. The work described in this thesis underlines the urgent need for greater research efforts to preserve the efficacy of INH for future generations.

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Appendix A

PATIENT INFORMATION SHEET

OXTREC 030 07

Testing for inclusion in study on drug resistant tuberculosis

(Screening for inclusion in Predictors of outcome in isoniazid resistant tuberculosis)

You are being considered for inclusion in a research study of drug resistant tuberculosis because you might have this condition. Please read this information sheet carefully or have someone read it to you. You will be given a copy of this form to keep.

What is the reason for screening?

We are looking for people who have tuberculosis that is resistant to the drug isoniazid and who do not have HIV infection to take part in a research study. You have already been diagnosed with tuberculosis and we would like to test your bacteria for resistance to isoniazid to see if you eligible for the study.

What will happen if I take part in the screening?

If you agree, we will test you for HIV infection and test your bacteria for resistance to isoniazid. If you fit the criteria for the study, we will invite you to join the study. You will be given more information about the study and you can decide whether to participate or not. There is no obligation to join the study if you fit the criteria. You will be given standard treatment for your condition, whether or not you decide to take part in the screening or the study. At any stage in the study you are completely free to withdraw your consent from the study. This will not affect the care you receive.

What tests will be done?

We will test you for :

1. HIV infection. If you are found to be HIV positive you can be referred to a clinic for HIV treatment if you wish.

2. If you agree we will also study your genes to try and understand why some people suffer from TB. This will involve studying the DNA from your cells. The DNA will be stored in a freezer for future studies of the genetic susceptibility to TB.

We will test your bacteria for:

1. resistance to isoniazid.

Your doctor will be given the results of these tests and will discuss them with you.

If you do not fit the criteria or if you decide not to participate in the study we will not store your samples.

What are the risks?

Having a blood test can be uncomfortable and may cause a bruise. Some people feel faint.

Confidentiality

Information about you will be kept confidential and will not be made available to anyone who is not connected with the study without your consent. All samples will be labeled with a study number rather than your name, to protect your identity.

Costs

You will not have to pay for anything other than the normal costs of routine care. All tests related to the study will be paid for.

Choosing or refusing to participate

You may refuse to be in any parts of the screening or the study. You can choose to consent to the screening study and not the genetics study if you want to. If you do not want to be screened that decision will not in any way interfere with your medical care or your ability to receive proper medical care or attention in the future.

Questions

If you have any other questions about the study please contact:

Doctor's name: Dr Phan Vuong Khach Thai

Telephone number:

PATIENT CONSENT FORM

OXTREC XXX

Testing for inclusion in study on drug resistant tuberculosis

(Screening for inclusion in Predictors of outcome in isoniazid resistant tuberculosis)

Consent from patient for isoniazid study screening

- ☐ **I have been fully informed of the possible risks and benefits of taking part in this screening and agree that I will take part.**
- ☐ **I know who to contact if I need more information. I understand that all information about me will be kept confidential. I understand that I am free to withdraw from the study at any time without affecting the care I will receive.**

Name _____ Signature: _____

Date_____

Consent from patient for isoniazid study screening and the genetics study

- ☐ **I have been fully informed of the possible risks and benefits of taking part in this screening and agree that I will take part.**
- ☐ **I agree that the samples may be stored and further tests undertaken (including genetic tests) in the future to further the understanding of this disease**
- ☐ **I know who to contact if I need more information. I understand that all information about me will be kept confidential. I understand that I am free to withdraw from the study at any time without affecting the care I will receive.**

Name _____ Signature: _____

Date_____

Investigator's statement

I, the undersigned, have defined and explained to the volunteer in a language he/she understands, the procedures of this study, its aims and the risks and benefits associated with his/her participation. I have informed the volunteer that confidentiality will be preserved, that he/she is free to withdraw from the trial at any time without affecting the care he/she will receive at the clinic. Following my definitions and explanations the volunteer agrees to participate in this study.

_____ _____

Date Name of investigator who gave the information about the study

Signature: _____

Appendix B

PATIENT INFORMATION SHEET

OXTREC XXX

Study number:_____

Results of treatment in tuberculosis resistant to the drug isoniazid

(Predictors of outcome in isoniazid resistant tuberculosis)

You are being being invited to take part in a research study of tuberculosis resistant to the drug isoniazid because you have this condition. Please read this information sheet carefully or have someone read it to you. You will be given a copy of this form to keep.

What is the reason for doing the study?

A lot of people in Viet Nam have tuberculosis that is resistant to the drug isoniazid. Although Isoniazid is a very important drug in the treatment of tuberculosis most people are successfully cured with normal tuberculosis treatment. We want to look at how resistance to isoniazid affects the success of tuberculosis treatment.

What will happen if I take part in the study?

You will be given standard treatment for your condition, whether or not you decide to take part in the screening or the study. At any stage in the study you are completely free to withdraw your consent from the study. This will not affect the care you receive.

What tests will be done?

During your visits to the clinic we will take some blood to test if you are infected with the virus hepatitis B or C and to see if you have any liver problems. These results will be given to your doctor and he can use the information to treat you for this condition if you have it. In addition to the routine hospital blood tests this study will involve taking an extra 14 mls blood (about 4 spoonfuls) on your first clinic visit and an extra 12mls at your 1 and 2 month visits.

We will also test your DNA for mutations in genes that may affect the way your body processes isoniazid.

Samples will be tested and stored indefinitely in a freezer at the Hospital for Tropical Diseases (HCMC). If you develop liver problems during your TB treatment we will do some

extra tests on the sample. Further tests may be done on stored samples in the future to improve our understanding of tuberculosis and hepatitis. The researcher doing these tests will not know your name, only your study code.

We will ask you to come to the clinic 3 times after you have finished your treatment for the doctor to see if your treatment was completely successful. You will be reimbursed for your travel for these visits so it will not cost you anything.

What are the risks?

Having a blood test can be uncomfortable and may cause a bruise. Some people feel faint.

Confidentiality

Information about you will be kept private and will not be made available to anyone who is not connected with the study without your consent. All samples will be labeled with a study number rather than your name, to protect your identity.

Costs

You will not have to pay for anything other than the normal costs of routine care. All tests related to the study will be paid for.

Choosing or refusing to participate

You may refuse to be in any parts of the study. If you do not want to take part that decision will not in any way interfere with your medical care or your ability to receive proper medical care or attention in the future.

Questions

If you have any other questions about the study please contact:

Doctor's name: Dr Phan Vuong Khach Thai

Telephone number:

PATIENT CONSENT FORM

OXTREC XXX

Testing for inclusion in study on drug resistant tuberculosis

(Screening for inclusion in Predictors of outcome in isoniazid resistant tuberculosis)

Consent from patient

☐ **I have been fully informed of the possible risks and benefits of taking part in this study and agree that I will take part.**

☐ **I know who to contact if I need more information. I understand that all information about me will be kept confidential. I understand that I am free to withdraw from the study at any time without affecting the care I will receive.**

Name _____ Signature: _____

Date_____

Investigator's statement

I, the undersigned, have defined and explained to the volunteer in a language he/she understands, the procedures of this study, its aims and the risks and benefits associated with his/her participation. I have informed the volunteer that confidentiality will be preserved, that he/she is free to withdraw from the trial at any time without affecting the care he/she will receive at the clinic. Following my definitions and explanations the volunteer agrees to participate in this study.

_____ _____

Date Name of investigator who gave the information about the study

Signature: _____

Action	Timepoint									
	presentation	enrolment	1	2	3	5	8	12	18	24
written consent to routine HIV test	x									
written consent to isoniazid resistance screening	x									
written consent to genetics study	x									
complete screening form for genetics	x									
2ml blood for genetics study	x									
written consent for full study		x								
complete enrolment form		x								
14mls blood		x								
hepatitis evaluation		x	x	x	x					
12 mls blood			x	x	x					
3 x sputum sample			x	x		x	x	x (if indicated)	x (if indicated)	x (if indicated)
document any adverse reactions			x	x	x	x	x			
document any treatment interruptions			x	x	x	x	x			
Document any additional medications			x	x	x	x	x			
TB symptomatic evaluation								x	x	x

Appendix C

SCREENING GENETICS
STUDY ENTRY FORM

PREDICTORS OF OUTCOME IN ISONIAZID RESISTANT TUBERCULOSIS

ENTRY DETAILS

1	SCREENING STUDY NUMBER	XXX
2	ADMISSION CENTRE	PNT ☐ Phu Nhuan ☐ District 2 ☐ District 4 ☐ Binh Thanh ☐
3	DATE PRESENTED AT ADMISSION CENTRE	_____/____/_____
4	DATE OF SCREENING	____/_____/_____

ENTRY CRITERIA

1	HIV test	positive ☐ negative ☐ unknown ☐
Patient cannot enter if HIV infected		
2	sputum smear result	positive ☐ negative ☐ unknown ☐
Patient cannot enter if smear negative		
3	Has the patient had previous Tb treatment	positive ☐ negative ☐ unknown ☐
Patient cannot enter if previously treated for TB		
4	Is patient ≥ 18 years of age	yes ☐ no ☐
patient cannot enter if < 18 years old		
5	Is patient pregnant?	yes ☐ no ☐
Patient cannot enter if pregnant		
6.	Will patient receive DOTS at this centre?	yes ☐ no ☐
Patient must intend to receive DOTS at study centre		
7	Has written informed consent been obtained?	yes ☐ no ☐
SIGNED CONSENT MUST BE OBTAINED BEFORE ENTRY INTO THE STUDY		

Appendix C

SCREENING GENETICS

SCREENING GENETICS ENTRY QUESTIONAIRE

Demographic Details

Full name_____

Age_____

Sex **Male** ☐ **Female** ☐

Ethnic Group_____

Occupation _____

Address_____

Days of illness _____

Radiology Date of X-ray_____

X-ray result	abnormal	Yes ☐	No☐
	consistent with active pulmonary TB	Yes ☐	No☐
	indeterminate	Yes ☐	No☐
	pleural effusion	Yes ☐	No☐
	pleural thickening	Yes ☐	No☐
	pneumothorax	Yes ☐	No☐
	miliary TB	Yes ☐	No☐
	pericardial involvement	Yes ☐	No☐

Any other evidence of extrapulmonary disease? **Yes** ☐ **No**☐
(eg lymphadenopathy, neck stiffness, headache, cranial nerve lesions)

Please take sample in accordance with the study schedule:
1. Genetics -2mls -EDTA tube

Appendix C

ENROLMENT

STUDY ENTRY FORM

PREDICTORS OF OUTCOME IN ISONIAZID RESITANT TUBERCULOSIS

ENTRY DETAILS

1	**INH RES STUDY NUMBER**	**XXX**
2	**ADMISSION CENTRE**	**PNT** ☐ Phu Nhuan ☐ District 2 ☐ District 4 ☐ Binh Thanh ☐
3	**DATE PRESENTED AT ADMISSION CENTRE**	_____/____/_____
4	**DATE ENROLLED TO STUDY**	____/_____/_____

ENTRY CRITERIA

1	**HIV test**	**positive** ☐ **negative** ☐ unknown ☐
Patient cannot enter if HIV infected		
2	**sputum smear result**	**positive** ☐ **negative** ☐ unknown ☐
Patient cannot enter if smear negative		
3	**Has the patient had previous Tb treatment**	**positive** ☐ **negative** ☐ unknown ☐
Patient cannot enter if previously treated for TB		
4	**Is patient ≥ 18 years of age**	**yes** ☐ **no** ☐
patient cannot enter if < 18 years old		
5	**Is patient pregnant?**	**yes** ☐ **no** ☐
Patient cannot enter if pregnant		
6.	**Will patient receive DOTS at this centre?**	**yes** ☐ **no** ☐
Patient must intend to receive DOTS at study centre		
7	**Has written informed consent been obtained?**	**yes** ☐ **no** ☐
SIGNED CONSENT MUST BE OBTAINED BEFORE ENTRY INTO THE STUDY		

Appendix C

ENROLMENT

ENTRY QUESTIONAIRE

Demographic Details

Full name_____

Age_____

Sex **Male** ☐ **Female** ☐

Ethnic Group_____

Occupation _____

Address_____

Telephone number for follow-up_____

Symptom Evaluation

Days of illness _____

Cough	Yes ☐	No ☐
Haemoptysis	Yes ☐	No ☐
Fever	Yes ☐	No ☐
Nightsweats	Yes ☐	No ☐
Weightloss (>10% bodyweight)	Yes ☐	No ☐
Chest pain	Yes ☐	No ☐
Malaise	Yes ☐	No ☐

Radiology

X-ray result No abnormalities ☐ Consistent with TB ☐ Indeterminate ☐

Appendix C

ENROLMENT

TB Contact History

Known contact with TB patient Yes ☐ No ☐

If yes, is it:

Family member Yes ☐ No ☐

Colleague Yes ☐ No ☐

Neighbour Yes ☐ No ☐

Other Yes ☐ No ☐

Past Medical History

Previous TB Yes ☐ No ☐

If yes state site: Pulmonary ☐

Extra-pulmonary ☐

BCG Vaccination Yes ☐ No ☐

BCG scar Yes ☐ No ☐

Jaundice Yes ☐ No ☐

Known Hepatitis infection Yes ☐ No ☐

Hep A ☐ Hep B ☐ HepC ☐ Hep D ☐ HepE ☐ unknown ☐

Renal Disease Yes ☐ No ☐

Diabetes Yes ☐ No ☐

Other illness Yes ☐ No ☐

If yes please specify _____

Social History

Smoker Yes ☐ No ☐

Drinks Alcohol Yes ☐ No ☐

If Yes state average weekly consumption _____Units/week
(1 beer = 1.5 units, 1 glass wine = 1.5 units, 1 whiskey = 1.5 units)

Previous heavy alcohol consumption? Yes ☐ No ☐

Medication History

Please list all current medications on next page

Appendix C

ENROLMENT

Hepatitis Evaluation

Risk factors for Hepatitis

Tattoos	Yes ☐	No ☐
IV drug use	Yes ☐	No ☐
Commercial sex worker	Yes ☐	No ☐
Man who has sex with men	Yes ☐	No ☐
Alcoholic	Yes ☐	No ☐
Health care worker	Yes ☐	No ☐
Previous Blood Transfusion	Yes ☐	No ☐
Haemophiliac	Yes ☐	No ☐

Examination Findings

Height _____cm Weight_____Kg

Cachexia	Yes ☐	No ☐
Lymphadenopathy	Yes ☐	No ☐
Anaemia	Yes ☐	No ☐
Signs of liver disease:		
Jaundice	Yes ☐	No ☐
Palmar erythema	Yes ☐	No ☐
Clubbing	Yes ☐	No ☐
Spider Naevi	Yes ☐	No ☐
Bruising	Yes ☐	No ☐
Gynaecomastia	Yes ☐	No ☐
Hepatomegaly	Yes ☐	No ☐
Splenomegaly	Yes ☐	No ☐
Caput Medusae	Yes ☐	No ☐
Ascites	Yes ☐	No ☐
Peripheral Oedema	Yes ☐	No ☐

If the patient has hepatitis has there been any confusion or loss of consciousness?
Yes ☐ No ☐

Appendix C

ENROLMENT

Please take samples in accordance with the study schedule:

1. INR – 2mls – Citrate tube (green)
2. Urea, electrolytes, creatinine & LFT's – 2mls – Lithium Heparin tube (black)
3. Serology – 4mls – Plain tube (red)
4. Viral load – EDTA – 4mls – EDTA tube (blue)
5. NAT2 testing -2mls -EDTA tube

DRUGS TAKEN BY PATIENT OTHER THAN STANDARD DOTS THERAPY

Please document any drugs taken by the patient other than standard DOTS therapy.

DRUG	REASON	DOSE/24HRS	ROUTE	START DATE	STOP DATE

Appendix C

Week 4 evaluation

Has the patient been diagnosed with hepatitis in the last 4 weeks?

Yes ☐ No ☐

Does the patient have any symptoms suggestive of hepatitis?

Eg	Nausea	Yes ☐	No ☐
	Vomiting	Yes ☐	No ☐
	Abdominal pain	Yes ☐	No ☐
	Anorexia	Yes ☐	No ☐
	Jaundice	Yes ☐	No ☐

Does the patient have any signs of hepatitis?

Jaundice	Yes ☐	No ☐
Palmar erythema	Yes ☐	No ☐
Spider Naevi	Yes ☐	No ☐
Bruising	Yes ☐	No ☐
Hepatomegaly	Yes ☐	No ☐
Ascites	Yes ☐	No ☐
Peripheral Oedema	Yes ☐	No ☐

List all medication taken in the last 4 weeks

_____

Has the patient drunk alcohol in the past 4 weeks?

Yes ☐ No ☐

If Yes state average weekly consumption _____Units/week
(1 beer = 1.5 units, 1 glass wine = 1.5 units, 1 whiskey = 1.5 units)

Please take samples in accordance with the study schedule:

1. INR – 2mls – Citrate tube
2. Urea, electrolytes, creatinine & LFT's – 2mls – Lithium Heparin tube
3. Serology – 4mls – Plain tube
4. Viral load – EDTA – 4mls – EDTA tube
5. 3 x sputum smear
6. 3 x LJ culture

Appendix C

Week 4 evaluation

ADVERSE EVENTS SHEET

Please complete if an adverse event has been noted at any time.

Describe event. eg. hepatitis/nausea/bleeding etc	**date of onset**	**Was treatment interupted?**	**date of interuption**	**drugs stopped** ***(list all drugs stopped)***	**restarted** ***(list start date for each individual drug)***

DRUGS TAKEN BY PATIENT OTHER THAN STANDARD DOTS THERAPY

Please document any drugs taken by the patient other than standard DOTS therapy.

DRUG	**REASON**	**DOSE/24HRS**	**ROUTE**	**START DATE**	**STOP DATE**

Appendix C

Week 8 evaluation

Has the patient been diagnosed with hepatitis in the last 4 weeks?

Yes ☐ No ☐

Does the patient have any symptoms suggestive of hepatitis?

Eg	Nausea	Yes ☐	No ☐
	Vomiting	Yes ☐	No ☐
	Abdominal pain	Yes ☐	No ☐
	Anorexia	Yes ☐	No ☐
	Jaundice	Yes ☐	No ☐

Does the patient have any signs of hepatitis?

Jaundice	Yes ☐	No ☐
Palmar erythema	Yes ☐	No ☐
Spider Naevi	Yes ☐	No ☐
Bruising	Yes ☐	No ☐
Hepatomegaly	Yes ☐	No ☐
Ascites	Yes ☐	No ☐
Peripheral Oedema	Yes ☐	No ☐

If the patient has hepatitis has there been any confusion or loss of consciousness?

Yes ☐ No ☐

List all medication taken in the last 4 weeks (excluding TB medication) on next page.

Has the patient drunk alcohol in the past 4 weeks? Yes ☐ No ☐

If Yes state average weekly consumption _____Units/week

(1 beer = 1.5 units, 1 glass wine = 1.5 units, 1 whiskey = 1.5 units)

Appendix C

Week 8 evaluation

Please take samples in accordance with the study schedule:

1. INR – 2mls – Citrate tube (green)
2. Urea, electrolytes, creatinine & LFT's – 2mls – Lithium Heparin tube (black)
3. Serology – 4mls – Plain tube (red)
4. Virol load – EDTA – 4mls – EDTA tube (blue)
5. 3 x sputum smear
6. LJ culture

Appendix C

Week 8 evaluation

ADVERSE EVENTS SHEET

Please complete if an adverse event is noted at any time.

Describe event. eg. hepatitis/nausea/bleeding etc	**date of onset**	**Was treatment interupted?**	**date of interuption**	**drugs stopped** ***(list all drugs stopped)***	**restarted** ***(list start date for each individual drug)***

DRUGS TAKEN BY PATIENT OTHER THAN STANDARD DOTS THERAPY

Please document any drugs taken by the patient other than standard DOTS therapy.

DRUG	**REASON**	**DOSE/24HRS**	**ROUTE**	**START DATE**	**STOP DATE**

Appendix C

Week 12 evaluation

Hepatitis evaluation

Has the patient been diagnosed with hepatitis in the last 4 weeks?

Yes ☐ No ☐

Does the patient have any symptoms suggestive of hepatitis?

Eg	Nausea	Yes ☐	No ☐
	Vomiting	Yes ☐	No ☐
	Abdominal pain	Yes ☐	No ☐
	Anorexia	Yes ☐	No ☐
	Jaundice	Yes ☐	No ☐

Does the patient have any signs of hepatitis?

Jaundice	Yes ☐	No ☐
Palmar erythema	Yes ☐	No ☐
Spider Naevi	Yes ☐	No ☐
Bruising	Yes ☐	No ☐
Hepatomegaly	Yes ☐	No ☐
Ascites	Yes ☐	No ☐
Peripheral Oedema	Yes ☐	No ☐

If the patient has hepatitis has there been any confusion or loss of consciousness?

Yes ☐ No ☐

List all medication taken in the last 4 weeks on next page.

Has the patient drunk alcohol in the past 4 weeks?

Yes ☐ No ☐

If Yes state average weekly consumption _____Units/week
(1 beer = 1.5 units, 1 glass wine = 1.5 units, 1 whiskey = 1.5 units)

Please take samples in accordance with the study schedule:

1. INR – 2mls – Citrate tube (green)
2. Urea, electrolytes, creatinine & LFT's – 2mls – Lithium Heparin tube (black)
3. Serology – 4mls – Plain tube (red)
4. Viral load – EDTA – 4mls – EDTA tube (blue)
5. 3 x sputum smear (if indicated)
6. 3 x LJ culture (if indicated)

Appendix C

ADVERSE EVENTS SHEET

Please complete if an adverse event is noted at any time.

Describe event. eg. hepatitis/nausea/bleeding etc	**date of onset**	**Was treatment interupted?**	**date of interuption**	**drugs stopped** ***(list all drugs stopped)***	**restarted** ***(list start date for each individual drug)***

DRUGS TAKEN BY PATIENT OTHER THAN STANDARD DOTS THERAPY

Please document any drugs taken by the patient other than standard DOTS therapy.

DRUG	**REASON**	**DOSE/24HRS**	**ROUTE**	**START DATE**	**STOP DATE**

Appendix C

20 week evaluation

Symptom Evaluation

Weight (kg) _____

Cough	Yes ☐	No ☐
Haemoptysis	Yes ☐	No ☐
Fever	Yes ☐	No ☐
Nightsweats	Yes ☐	No ☐
Weightloss (>10% bodyweight)	Yes ☐	No ☐
Chest pain	Yes ☐	No ☐
Malaise	Yes ☐	No ☐

Please take samples in accordance with the study schedule:

1. 3x sputum smear.
2. 3 x LJ culture

Appendix C

8 month evaluation

Symptom Evaluation

Weight (kg) _____

Cough	Yes ☐	No ☐
Haemoptysis	Yes ☐	No ☐
Fever	Yes ☐	No ☐
Nightsweats	Yes ☐	No ☐
Weightloss (>10% bodyweight)	Yes ☐	No ☐
Chest pain	Yes ☐	No ☐
Malaise	Yes ☐	No ☐

If the answer is yes to any of the questions above, please take X-ay and 3x sputum for smear and culture

X-ray result No abnormalities ☐ Consistent with TB ☐ Indeterminate ☐

Has their been any evidence of hepatitis throughout the course of treatment?	Yes ☐ No ☐	

Appendix C

8 month evaluation

8 MONTH OUTCOME ON COMPLETION OF TREATMENT (by WHO definitions)

Please tick one at 8 month completion of treatment

Outcome	Definition	
Cured	A patient who was initially smear positive and who was smear negative in the last month of treatment and on at least one previous occasion	
Completed treatment	A patient who completed treatment but did not meet the criteria for cure or failure.	
Died	A patient who died from any cause during treatment	
Failed	A patient who was initially smear positive and who remained smear positive at month 5 or later during treatment.	
Defaulted	A patient who transferred to another reporting unit and for whom the treatment outcome is not known.	

Appendix C

8 month evaluation

ADVERSE EVENTS SHEET
Please complete if an adverse event is noted at any time.

Describe event. eg. hepatitis/nausea/bleeding etc	**date of onset**	**Was treatment interupted?**	**date of interuption**	**drugs stopped** *(list all drugs stopped)*	**restarted** *(list start date for each individual drug)*

Appendix C

12 month evaluation

Symptom Evaluation

Weight (kg) _____

Cough	Yes ☐	No ☐
Haemoptysis	Yes ☐	No ☐
Fever	Yes ☐	No ☐
Nightsweats	Yes ☐	No ☐
Weightloss (>10% bodyweight)	Yes ☐	No ☐
Chest pain	Yes ☐	No ☐
Malaise	Yes ☐	No ☐

If the answer is yes to any of the questions above, please take X-ay and 3x sputum for smear and culture

X-ray result No abnormalities ☐ Consistent with TB ☐ Indeterminate ☐

Appendix C

18 month evaluation

Symptom Evaluation

Weight (kg) _____

Cough	Yes ☐	No ☐
Haemoptysis	Yes ☐	No ☐
Fever	Yes ☐	No ☐
Nightsweats	Yes ☐	No ☐
Weightloss (>10% bodyweight)	Yes ☐	No ☐
Chest pain	Yes ☐	No ☐
Malaise	Yes ☐	No ☐

If the answer is yes to any of the questions above, please take X-ay and 3x sputum for smear and culture

X-ray result No abnormalities ☐ Consistent with TB ☐ Indeterminate ☐

Appendix C

24 month evaluation

Symptom Evaluation

Weight (kg) _____

Cough	Yes ☐	No ☐
Haemoptysis	Yes ☐	No ☐
Fever	Yes ☐	No ☐
Nightsweats	Yes ☐	No ☐
Weightloss (>10% bodyweight)	Yes ☐	No ☐
Chest pain	Yes ☐	No ☐
Malaise	Yes ☐	No ☐

If the answer is yes to any of the questions above, please take X-ay and 3x sputum for smear and culture

X-ray result No abnormalities ☐ Consistent with TB ☐ Indeterminate ☐

Appendix C

Patient name _____

DTU centre_____ **DTU telephone** _____

DOTS ADMINISTRATION RECORD

Please tick each day drugs are taken. Record any missing or incomplete doses on page x.
Month 1 + 2 to be completed by DTU staff. Month 3-8 to be completed by patient.

Month 1. Start date of treatment ____/____/____

Day		1	2	3	4	5	6	7
Week								
1								
2								
3								
4								

Month 2 Start date _____/____/_____

Day		1	2	3	4	5	6	7
Week								
1								
2								
3								
4								

Appendix C

Patient name _____

DTU centre_____ **DTU telephone** _____

Please cross each day that you take your TB medicine. If you forget to take your medicine please do not tick the box so that the record is accurate. Please give this back to the nurse each time you return for your check-up.

Month 3 Start date ____/____/____

Day		1	2	3	4	5	6	7
Week								
1								
2								
3								
4								

Please go to the DTU at the end of 3 months for your check-up

Month 4. Start date ____/____/____

Day		1	2	3	4	5	6	7
Week								
1								
2								
3								
4								

Month 5. Start date ___/___/____

Day		1	2	3	4	5	6	7
Week								
1								
2								
3								
4								

Please go to the DTU at the end of 5 months for your check-up

Appendix C

Patient name _____

DTU centre_____ **DTU telephone** _____

Month 6. Start date ____/____/_____

Day		1	2	3	4	5	6	7
Week								
1								
2								
3								
4								

Month 7. Start date ____/____/_____

Day		1	2	3	4	5	6	7
Week								
1								
2								
3								
4								

Month 8. Start Date ____/____/_____

Day		1	2	3	4	5	6	7
Week								
1								
2								
3								
4								

Please go to the DTU at the end of 8 months for your check-up

Appendix C

INVESTIGATION RESULT SHEET

TB microbiology

sputum smear	timepoint	sample date	result
	0		
	4 weeks		
	8 weeks		
	20 weeks		
	8 months		
	12 months		
	18 months		
	24 months		
LJ culture	0		
	4 weeks		
	8 weeks		
	20 weeks		
	8 months		
	12 months		
	18 months		
	24 months		

Biochemistry

investigation	date	result	units
Na			mmol/L
K			mmol/L
Urea			mmol/L
Creatinine			µmol/L
ALT			I.U./L
AST			I.U./L
protein			g/L
bilirubin			µmol/L
INR (if done)			

Appendix C

Serology

HBsAg	**Positive**	☐	Negative☐
HBcAb	**Positive**	☐	Negative☐
HCVAb	**Positive**	☐	Negative☐

Hepatits B viral load (if positive) _____copies/ml

Hepatitis C Viral load (if positive) _____copies/ml